Amplification of Telomeric Arrays via Rolling-circle Mechanism*

Received for publication, August 13, 2004, and in revised form, December 17, 2004
Published, JBC Papers in Press, January 18, 2005, DOI 10.1074/jbc.M409295200

Jozef Nosek‡, Adriana Rycovska‡, Alexander M. Makhov§, Jack D. Griffith§§, and Lubomir Tomaska§§‡‡

From the ‡Department of Biochemistry, Mlynska dolina CH-1, and the §Department of Genetics, Mlynska dolina B-1, Faculty of Natural Sciences, Comenius University, 842 15 Bratislava, Slovakia and §§Lineberger Comprehensive Cancer Center and Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27599

Alternative (telomerase-independent) lengthening of telomeres mediated through homologous recombination is often accompanied by a generation of extrachromosomal telomeric circles (t-circles), whose role in direct promotion of recombinational telomere elongation has been recently demonstrated. Here we present evidence that t-circles in a natural telomerase-deficient system of mitochondria of the yeast Candida parapsilosis replicate independently of the linear chromosome via a rolling-circle mechanism. This is supported by an observation of (i) single-stranded DNA consisting of concatameric arrays of telomeric sequence, (ii) lasso-shaped molecules representing rolling-circle intermediates, and (iii) preferential incorporation of deoxyribonucleotides into telomeric fragments and t-circles. Analysis of naturally occurring variant t-circles revealed conserved motifs with potential function in driving the rolling-circle replication. These data indicate that extrachromosomal t-circles observed in a wide variety of organisms, including yeasts, plants, Xenopus laevis, and certain human cell lines, may represent independent replicons generating telomeric sequences and, thus, actively participating in telomere dynamics. Moreover, because of the promiscuous occurrence of t-circles across phyla, the results from yeast mitochondria have implications related to the primordial system of telomere maintenance, providing a paradigm for evolution of telomeres in nuclei of early eukaryotes.

Telomeres, specialized nucleoprotein structures at the ends of linear DNA molecules, are crucial for both proper replication of the chromosomal ends and their protection against inappropriate DNA repair, end-to-end fusions, and exonucleolytic degradation (1, 2). Although telomerase, the enzyme responsible for telomere maintenance in most circumstances, has received the most attention (3, 4), there is increasing interest in telomere maintenance in most circumstances, including yeast mutants deleted for telomerase, native chromosomal or mitochondrial telomeres in some species, and a significant minority of human cancers, where the phenomenon is called Alternative Lengthening of Telomeres (10). Extrachromosomal copies of telomeric sequence, often known to be in a circular form (designated t (telomeric)-circles),1 have been found in a wide variety of organisms, including yeasts, plants, amphibians, and certain mammalian cells (9). Originally, they were related to endogenous and induced genomic instability in mammalian cells (11). Later, results from our laboratories indicated that t-circles are active players in telomere maintenance (12). In the case of Kluyveromyces lactis, t-circles have been shown to directly promote recombinational telomere elongation (13, 14). In addition, synthetic DNA nanocircles composed of human telomeric repeats can act as essentially infinite catalytic templates for efficient synthesis of long telomeres by conventional DNA polymerase (15).

A role of t-circles in telomere maintenance was originally proposed for a natural telomerase-deficient system of mitochondria of the yeast Candida parapsilosis (12). The ends of its linear mitochondrial genome consist of tandem arrays of a 738-bp-long repetitive unit and a 5′ single-stranded overhang of about 110 nucleotides (Fig. 1A) (16). Two-dimensional agarose gel electrophoretic and electron microscopic (EM) analyses demonstrated the presence of minicircular DNA molecules derived exclusively from the telomeric sequence (12). Importantly, two recent papers demonstrated that human alternative lengthening of telomere (ALT) cells have abundant t-circles, pointing to their potential role in promoting telomere replication in the absence of telomerase (17, 18). However, experimental evidence that naturally occurring t-circles are employed as substrates for amplification of telomeric arrays in vivo is lacking.

Mitochondrial t-circles are present as series of integral multiples of the tandem repeat unit. It was suggested that they are not only by-products of recombination between telomeric tandem repeat units but that they also may serve as templates to promote recombinational telomere elongation. This would imply that the t-circles amplify independently of the main mitochondrial chromosome, leading to de novo generation of extrachromosomal telomeric sequences that would be integrated back at the mtDNA termini. To test this hypothesis, we employed two-dimensional gel electrophoresis and EM to search for intermediates generated during replication of the mitochondrial t-circles. In this report we provide the first evidence that t-circles replicate via a rolling-circle strategy, thus pointing to their active partici-

* This work was supported in part by grants from the Fogarty International Research Collaboration (1-R03-TW05654-01), Howard Hughes Medical Institute (55000327), and the Slovak grant agencies VEGA (1/2331/05 and 1/0006/03) and APVT (20–003902). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: t-circle, telomeric circle; EM, electron microscopy; ssDNA, single-stranded DNA.

1 The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBankTM/EBI Data Bank with accession number(s) AF463777, AF463778, and AF463779. Supported by an Ellison Senior Scholar Award and National Institutes of Health Grant GM31819.

** To whom correspondence should be addressed. Tel.: 421-2-60296-536; Fax: 421-2-60296-452; E-mail: tomaska@fns.uniba.sk.

Printed in U.S.A.

This paper is available on line at http://www.jbc.org

10840
pation in the dynamics of telomeric tandem repeats and amplification of extrachromosomal telomeric arrays in vivo. In addition, these results have implications for evolution of telomeric tandem arrays in chromosomes of early eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Yeast Strain and Oligonucleotides—** *C. parapsilosis SR23 (CBS 7157)* is a laboratory strain from the collection of the Department of Biochemistry, Comenius University, Bratislava, Slovakia. Strains *MC0447* and *MC0448* were kindly provided by P. F. Lehmann (Medical College of Ohio, Toledo). TELS1 (5′-TAGGATGATTATTTAATATATTAT-CAATTATATAAACGATAATA-3′) represents the sequence of the last 51 nucleotides from the 5′-overhang of the extreme end of *C. parapsilosis* mtDNA (16), and Oligo2 (5′-GTATTTATCTTTCTTATTAGAAC-3′) is derived from the same strand of the telomeric repeat unit. TELS1C and Oligo2C are complementary to TELS1 and Oligo2, respectively (Fig. 1, A and C).

**DNA Manipulations—** *C. parapsilosis* was grown in YPD medium (1% yeast extract, 1% peptone, 2% glucose) on a rotary shaker at 30 °C to mid-log phase. The cells were then harvested, resuspended in 0.1% sodium azide, 100 mM EDTA, pH 8.0, and incubated for 10 min on ice. Subsequently, the cells were collected by centrifugation and total DNA was isolated as described (19). Neutral-neutral two-dimensional agarose gel electrophoresis was performed by the method of Brewer and Fangman (20) with slight modifications outlined in Ref. 12. Enzymatic DNA manipulations, DNA cloning, Southern blotting, and DNA labeling and hybridization were performed according to Ref. 21. The t-circles were cloned as restriction enzyme fragments (EcoRI (SR23; 738 bp), EcoRV (MC0448; 620 bp), and ClaI (MC0471; 777 bp)) into corresponding sites of pUC/pTZ plasmid vectors, and their nucleotide sequences were determined by the dideoxy chain termination method. The nucleotide sequences have been submitted to the GenBank™ database with accession numbers AY468377 (SR23), AY468378 (MCO448), and AY468379 (MCO471).

**In Organello Replication Assay—** Freshly prepared mitochondria (0.3 ml) (12) were mixed on ice with 40 μl of buffer L (250 mM Tris-HCl (pH 7.4), 100 mM pyruvate, 100 mM sodium phosphate (pH 7.4), 10 mM MgCl2, 40 μl of 0.8 M sucrose, 10 μl of 100 mM MgCl2, 40 μl of 20 mM ATP, and 40 μl of 150 μM dTTP, dGTP, dATP). The reaction was started by addition of 5 μl of [α-32P]dCTP and immediately placed in a 22 °C waterbath. 90-ml aliquots were taken at 0, 2, 5, 10, and 15 min, mixed with 90 μl of 2× mito-lysing buffer (300 mM NaCl, 20 mM Tris-HCl (pH 7.4), 50 mM EDTA-NaOH (pH 8.0), 2% sarcosyl) and incubated for 10 min at 65 °C followed by 10 min on ice. After addition of 220 μl of 1× mito-lysing buffer, 2 μl of proteinase K (20 mg/ml) were added and incubated for 60 min at 37 °C. Each sample was extracted once with phenol:chloroform (1:1), followed by incubation with 50 μg/ml RNase A for 15 min at 37 °C. The samples were extracted twice with phenol:chloroform:isoamylalcohol (25:24:1). DNA was ethanol precipitated and the vacuum-dried pellet was solubilized in 4 ml of TE (10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA-NaOH). The resulting samples were diluted with two volumes of TE, and DNA was precipitated with ethanol and solubilized in 150 μl of TE. 20 μl of the resulting DNA samples were used for Southern blot hybridization and EM.

**Electron Microscopy—** Preparation of the DNAs for EM is described in Refs. 22 and 23. A Phillips CM12 was used at 40 kV. Images on film were scanned using a Nikon LS4500 film scanner and the contrast adjusted using Adobe Photoshop software. *Escherichia coli* SSB protein was purified as described (24). A plasmid pGEMEX-1 (Invitrogen) was used as an internal standard.

**RESULTS AND DISCUSSION**

When undigested DNA of *C. parapsilosis* is subjected to neutral-neutral two-dimensional gel electrophoresis, there is a relatively high number of circular double-stranded DNA molecules composed of exclusively telomeric sequences whose sizes correspond to integral multiples of the telomeric tandem repeat unit (738 bp). This is exemplified in Fig. 1B, where hybridization was performed with the probe derived from a 738-bp EcoRI restriction fragment corresponding to the telomeric repeat unit. In addition to double-stranded t-circles, the hybridization revealed the presence of an additional class of fast-migrating DNA molecules of heterogeneous sizes (Fig. 1B). These DNAs were also present when the blot was hybridized with the oligonucleotides TELS1C and Oligo2C derived from two different tandem arrays in chromosomes of early eukaryotes.
regions of the telomeric unit and, thus, t-circle sequence. In contrast, the fast-migrating DNA species were absent when the same blot was probed with complementary oligonucleotides TEL51 and Oligo2. The fact that these molecules are detected only with TEL51C/Oligo2C and not with TEL51/Oligo2 suggests that they (i) have a single-stranded nature, (ii) are derived from the same strand of DNA, and (iii) might represent replication intermediates that would be typically generated by a strand displacement synthesis such as rolling-circle replication (Fig. 1C).

To obtain samples that would be enriched for these intermediates, we employed a propidium iodide-CsCl gradient that was originally used for buoyant separations of various forms of \( \phi X174 \) DNA (25). The fractions from the gradient were subjected to Southern blot analysis with the TEL51 and TEL51C probes (Fig. 2A). Double-stranded telomeric DNA circles hybridizing to both probes were found within the lower part of the tube (Fig. 2A, lanes 2 and 3). Molecules with higher buoyant density contained mostly ssDNAs hybridizing to TEL51C (Fig. 2A, lanes 6 and 7). A small portion of the molecules in these fractions hybridized to both oligonucleotides, suggesting their double-stranded nature (Fig. 2A, lane 7). The length of the DNAs was highly heterogeneous, starting at \(-0.75\) kbp (the size of the smallest t-circle) and reaching several kbp.

To directly visualize DNA molecules present in the fraction with high buoyant density, the sample was analyzed by EM. The single-stranded regions were stained with ssDNA-binding protein from \( E. \ coli \). As indicated by Southern blot analysis (Fig. 2A), the fraction contained a high concentration of long ssDNA molecules of various sizes (data not shown). More importantly, about 50 lasso-shaped double-stranded DNA molecules with a single-stranded region at the junction between the circle and the tail (Fig. 2B) were scored. The other fractions from the gradient did not contain this type of molecule.

The structures observed could be due to various types of DNA transactions. For example, the unique recombinational single-step deletion process termed telomere rapid deletion may shorten telomeres through an intratelomeric loop intermediate (7, 26). Another related possibility might be a strand invasion to form a protective t-loop structure (23, 27), which might even occur with extrachromosomal telomeric pieces. However, the heterogeneous sizes of the “tails”, relatively large ssDNA regions at the circle/tail junction, and observation of the structures in preparations not treated with psoralen (required to visualize telomeric loops) strongly argue that the molecular architecture observed represents a typical intermediate of rolling-circle replication (28).

The above results indicate that the telomeric region of mtDNA of \( C. \ parapsilosis \) is highly dynamic and might represent a hot spot for DNA transactions such as DNA replication and/or recombination. To address this hypothesis experimentally, we performed in \( \text{organello} \) DNA replication assays on purified \( C. \ parapsilosis \) mitochondria. After initiation of the reaction by addition of \( [\alpha-^{32}\text{P}]dCTP \), aliquots were taken at 0, 2, 5, 10, and 15 min, and DNA was isolated and digested with BglIII, HindIII, PvuII, and XbaI endonucleases, allowing discrimination between telomeric and internal restriction enzyme fragments of the mtDNA (Fig. 3A). Autoradiography of a conventional agarose gel with separated mtDNA fragments revealed that the majority of the radioactive label was incorporated into the telomeric regions of the mtDNA (Fig. 3, B and C). Importantly, two-dimensional electrophoresis of the BglIII-
digested mtDNA revealed two labeled populations of DNA fragments corresponding to t-circles and a fast-migrating ssDNA species, respectively, suggesting that displacement DNA synthesis of t-circles is involved in de novo synthesis of telomeric DNA (Fig. 3D). Although the proportion of labeled t-circles is relatively low (indicating their slow de novo formation), there is a high abundance of fast-migrating DNA species (reflecting a high rate of rolling-circle amplification). The DNA species observed are similar to those detected by Southern blot analysis of BglII-digested mtDNA hybridized with an EcoRI-EcoRI telomeric fragment (Fig. 3E). These results demonstrate that mitochondrial telomeres of C. parapsilosis are preferential sites of de novo DNA synthesis and/or recombination, which is in line with a potential role of rolling-circle replication of t-circles in telomere maintenance.

A recent survey of several strains of C. parapsilosis revealed that an interspecific variability affects the sequence of the telomeric tandem repeat motifs (29). In contrast to the strain SR23 employed in the present study, the telomeric repeat in the strain MCO448 is significantly shorter (620 bp in MCO448 versus 738 bp in SR23). To demonstrate that the size of the telomeric repeat corresponds to the size of the t-circles, circular DNA molecules were isolated from mitochondria of MCO448 by the alkaline lysis method (12) and subjected to EM analysis. The population of purified t-circles consisted of DNA molecules whose size ranged between ~600 and 5600 bp, corresponding to 1–9 multimers of the 620-bp-long telomeric repeat (Fig. 4).
Next, in silico analysis was performed to define critical regions responsible for the dynamics of t-circles of C. parapsilosis. It revealed the presence of four palindromic sequences that may be involved in promoting rolling-circle replication (Fig. 5A). Interestingly, the position of the 5'-end of the mitochondrial chromosome corresponds to the base of palindrome 3, which may represent a potential site for a specific nick during opening of a t-circle prior to rolling-circle synthesis. Moreover, the t-circle sequence contains a cytosine-rich domain (C-box) that is a candidate for a hot spot implicated in recombinational transactions at telomeric arrays. Furthermore, a guanine-rich domain (G-box) localized at the boundary of the 554-bp subterminal repeat and the tandem array (Fig. 1A) may be also implicated in these events.

To identify general features of the t-circles that might be important for their function, we cloned and sequenced t-circles from the strains of C. parapsilosis differing in size of telomeric repeats and corresponding t-circles (i.e., 738 bp in SR23, 620 bp in MCO448, and 777 bp in MCO471). A comparison of their sequences (Fig. 5B) revealed that all four palindromes and the C-box found in the strain SR23 are highly conserved among all three variants, indicating biological significance of these mo-
tifs. Covariation of bases in palindrome 1 further supports this idea.

In addition to the strains with altered sequence of the terminal tandem repeat, the survey of C. parapsilosis isolates revealed mutants with a circularized form of the mitochondrial genome. The conversion of linear to circular mtDNA was due to fusion of termini of the linear molecules that was accompanied by deletion of a significant fraction of the telomeric sequence with a concomitant loss of mitochondrial t-circles (29). Elimination of t-circles might have caused a defect in the telomere maintenance pathway that has been evaded by circularization of the genophore. This would imply that the t-circle-dependent pathway may represent the main, or even the only, mode of telomere maintenance in C. parapsilosis mitochondria.

These results, together with studies on nuclear telomeres of K. lactis and Xenopus laevis (13, 30), imply that t-circles are not simply by-products of recombination transactions within the tandem arrays but play an important role in a telomere maintenance pathway. Rolling-circle replication of the t-circles generates elongated stretches of telomeric repeats that may be incised back at the chromosomal ends. A wide occurrence of t-circles in both nuclear and mitochondrial compartments argues that an excision-expansion-incision cycle undergone by t-circles provides a general, telomerase-independent mode of telomere maintenance (9). Replication of linear mitochondrial genomes (31) therefore provides a paradigm for evolution of telomeres of eukaryotic chromosomes.

Considering their eubacterial origin (32), mitochondria of C. parapsilosis, as a natural telomerase-deficient system of telomere replication, may be solving the end-replication problem by an evolutionary ancient mechanism preceding telomerase. This is in line with a recent hypothesis that nuclear telomere termini were originally mediated by telomeric loops (37). Expanded telomeric arrays subsequently might have allowed formation of the telomeric loop structures. Telomerase might have come later, outcompeted ancient mechanism(s), and provided a more robust mechanism for the maintenance of telomeres in eukaryotic nuclei. Therefore, in addition to their significance for understanding the details of alternative lengthening of telomere pathways, the results presented here have evolutionary implications related to the prymordial system of nuclear telomere maintenance.

Acknowledgments—We thank Ladislav Kovac (Comenius University, Bratislava) for continuous support and helpful comments, members of our laboratories for discussions, and Judita Slezakova for technical assistance.

REFERENCES

1. McEachern, M. J., Krauskopf, A., and Blackburn, E. H. (2000) Ann. Rev. Genet. 255, 331–358
2. de Lange, T. (2002) Oncogene 21, 532–549
3. Greider, C. W., and Blackburn, E. H. (1985) Cell 43, 405–413
4. Bryan, T. M., and Cech, T. R. (1999) Curr. Opin. Cell Biol. 11, 318–324
5. Lushbld, V. (2002) Oncogene 21, 522–531
6. Reddel, R. R. (2003) Cancer Lett. 194, 155–162
7. Lustig, A. J. (2003) Nat. Rev. Genet. 4, 916–923
8. de Lange, T. (2004) Nat. Rev. Mol. Cell. Biol. 5, 323–329
9. Tomaska, L., McEachern, M. J., and Nosek, J. (2004) FEBS Lett. 567, 142–146
10. Reddel, R., and Bryan, T. M. (2005) Lancet 361, 1849–1841
11. Regev, A., Cohen, S., Cohen, E., Bar-Am, I., and Lavi, S. (1998) Oncogene 17, 3455–3461
12. Tomaska, L., Nosek, J., Makov, A. M., Pastarokova, A., and Griffith, J. D. (2000) Nucleic Acids Res. 28, 4479–4487
13. Natarajan, S., and McEachern, M. J. (2002) Mol. Cell. Biol. 22, 4512–4521
14. Natarajan, S., Grof-Vindman, C., and McEachern, M. J. (2003) Eukaryot. Cell 2, 1115–1127
15. Lindstrom, U. M., Chandrasekaran, R. A., Orbai, L., Helquist, S. A., Miller, G. P., Oroudevj, E., Hansma, H. G., and Kool, E. T. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15953–15958
16. Nosek, J., Dinouel, N., Kovac, L., and Fukuhara, H. (1995) Mol. Gen. Genet. 247, 61–72
17. Cesare, A. J., and Griffith, J. D. (2004) Mol. Cell. Biol. 24, 9948–9957
18. Wang, R. C., Smogorzewska, A., and de Lange, T. (2004) Cell 119, 355–368
19. Philippens, P., Stotz, A., and Scherf, C. (1991) Methods Enzymol. 194, 169–182
20. Brewer, B. J., and Fangman, W. L. (1988) Cell 55, 637–643
21. Sambrook, J., and Russell, D. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
22. Griffith, J. D., and Christiansen, G. (1978) Annu. Rev. Biophys. Bioeng. 7, 19–35
23. Griffith, J. D., Comeau, L., Rosenfeld, S., Stansel, R. M., Bianchi, A., Moss, H., and de Lange, T. (1999) Cell 97, 503–514
24. Chase, J. W., Whitier, R. F., Auerbach, J., Sancar, A., and Rupp, W. D. (1980) Nucleic Acids Res. 8, 3215–3227
25. Fukuda, A. (1976) J. Biochem. 80, 253–258
26. Li, B., and Lustig, A. J. (1995) Science 301, 1310–1326
27. Tomaska, L., Makov, A. M., Griffith, J. D., and Nosek, J. (2002) Mitochon- drion 1, 445–459
28. Kornberg, A., and Baker, T. A. (1992) DNA Replication, pp. 298–300, 2nd Ed., W. H. Freeman & Co., New York
29. Rykovska, A., Valach, M., Tomaska, L., Bolotin-Fukuhara, M., and Nosek, J. (2004) Microbiology (UK) 150, 1571–1590
30. Cohen, S., and Mehalli, M. (2002) EMBO Rep. 3, 1168–1174
31. Nosek, J., Tomaska, L., Fukuhara, H., Suyama, Y., and Kovac, L. (1998) Trends Genet. 14, 184–188
32. Martin, W., and Russell, M. J. (2003) Philos. Trans. R. Soc. Lond. B. Biol. Sci. 358, 59–83
33. Tomaska, L., Nosek, J., and Fukuhara, H. (1997) J. Biol. Chem. 272, 3049–3056
34. Nosek, J., Tomaska, L., Pagacova, B., and Fukuhara, H. (1999) J. Biol. Chem. 274, 8850–8857
35. Maiuzis, N., and Weiner, A. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6729–6734
36. Nosek, J., and Tomaska, L. (2002) in Telomeres, Telomerases, and Cancer (Krupp, G., and Parwaresch, R., eds), pp. 396–417, Kluwer Academic/Plenum Publishers, New York
37. Nosek, J., and Tomaska, L. (2003) Curr. Genet. 44, 73–84
38. Nosek, J., Novotna, M., Hlavatovicova, Z., Ussery, D. W., Fajkus, J., and Tomaska, L. (2004) Mol. Genet. Genomics 272, 173–180