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Amplification of Telomeric Arrays via Rolling-circle Mechanism*

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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 elongation mediated through telomerase-independent mechanisms (5–9). Recombination appears to maintain telomeres in a wide variety of 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. 1*A*) (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-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AY468377, AY468378, and AY468379.

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

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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 MCO471 and MCO448 were kindly provided by P. F. Lehmann (Medical College of Ohio, Toledo). TEL51 (5′-TAGGGATTGATTATTTACCTATATATTATCAATTATATTAAACGATAATA-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′-GTATTTATTTCTTATTCATTCTTCATTAGAAC-3′) is derived from the same strand of the telomeric repeat unit. TEL51C and Oligo2C are complementary to TEL51 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 (MCO448; 620 bp), and ClaI (MCO471; 777 bp)) into corresponding sites of pUC/pTZ plasmid vectors, and their sequences were determined by the dideoxy chain termination method. The nucleotide sequences have been submitted to the GenBank™ 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 NADH, 10 mM malate, 20 mM KCl), 40 μl of 0.8 M sucrose, 40 μl of 100 mM $MgCl_2$, 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-^{32}P]dCTP and immediately placed in a 22 °C waterbath. 90-μl aliquots were taken at 0, 2, 5, 10, and 15 min, mixed with 90 μl of 2× mito-lysis 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-lysis 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 50 μl of double-distilled water. The samples (7 μl) were digested with a restriction enzyme, and resulting fragments were separated either by one-dimensional electrophoresis in 1% agarose gel (21) or two-dimensional electrophoresis (12). The gels were stained with ethidium bromide, dried, and subjected to autoradiography.

Fractionation of mtDNA on Propidium Iodide-CsCl Gradient—The mitochondrial pellet (0.5 ml) was lysed with 4.5 ml of the lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA-NaOH, 1% sarcosyl) and incubated for 10 min on ice. After addition of proteinase K (Sigma) to a final concentration of 200 μg/ml, the suspension was incubated for 30 min at 37 °C, followed by two extractions with equal volume of phenol:chloroform:isoamylalcohol (25:24:1). The deproteinized sample was treated for 20 min at 37 °C with RNase A (100 μg/ml) and re-extracted with phenol:chloroform:isoamylalcohol. The nucleic acids were precipitated with ethanol, and the resulting pellets were dried under vacuum and solubilized in 4 ml of TE (10 mM Tris-HCl (pH 7.4), 1 mM EDTA-NaOH) containing 0.6 mg/ml propidium iodide. CsCl (3.34 g) was added, and the sample was centrifuged for 23 h at 55,000 rpm in a T863B rotor at 15 °C. Fractions (0.5 ml) were collected from the bottom of the tube. Propidium iodide was removed by Dowex A50 (Sigma) (21). The resulting samples were diluted with two volumes of TE, and DNA was precipitated with ethanol and solubilized with 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 Philips 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.

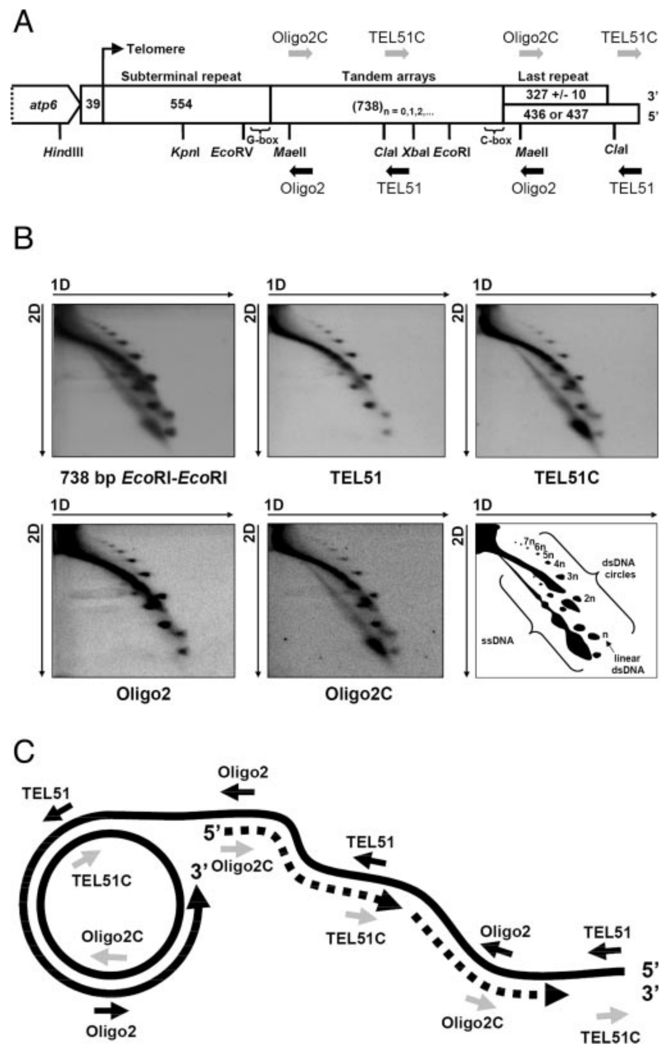


FIG. 1. **Replication of t-circles in mitochondria of *C. parapsilosis* involves displacement DNA synthesis.** *A*, organization of the mitochondrial telomeres of *C. parapsilosis* strain SR23 and position of the probes TEL51/TEL51C and Oligo2/Oligo2C. *B*, mitochondrial DNA of *C. parapsilosis* was separated using two-dimensional gel electrophoresis, followed by Southern blot hybridization using a radioactively labeled 738-bp EcoRI-EcoRI telomeric fragment, TEL51, TEL51C, Oligo2, and Oligo2C. The *scheme* summarizes DNA species separated by two-dimensional gel electrophoresis. *C*, the structure of putative t-circle replication intermediate illustrating displacement of the 5′-strand and synthesis of the lagging strand, presumably via Okazaki fragments. Positions of oligonucleotides TEL51/Oligo2 and TEL51C/Oligo2C are shown as *black* and *gray arrows*, respectively.

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. 1*B*, 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. 1*B*). These DNAs were also present when the blot was hybridized with the oligonucleotides TEL51C and Oligo2C derived from two different

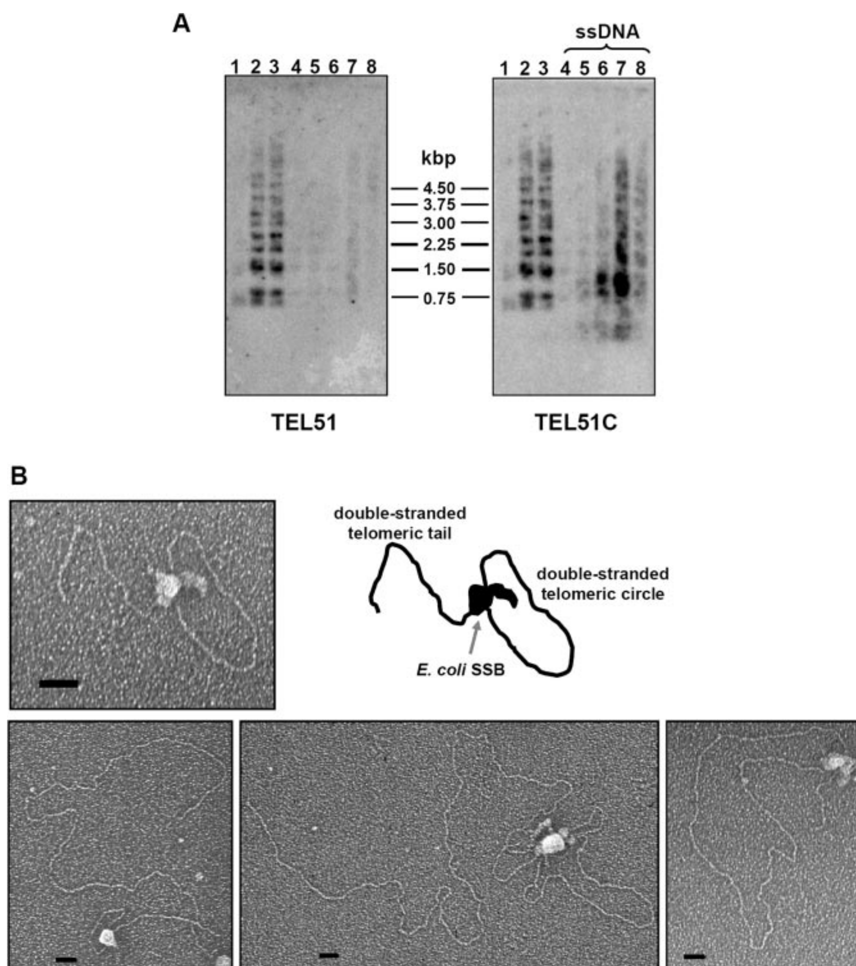


FIG. 2. **Lasso-shaped molecules in a fraction of mtDNA prepared using a propidium iodide-CsCl gradient.** *A*, fractionation of mtDNA of *C. parapsilosis* strain SR23 using a propidium iodide-CsCl gradient was followed by agarose gel electrophoresis and Southern blot hybridization with TEL51 and TEL51C oligonucleotide probes. *B*, examples of molecular species in fraction 7 from the propidium iodide-CsCl gradient observed by EM. *Bar* represents 0.1 μm. The *scheme* illustrates the architecture of a rolling-circle indicating the position of a ssDNA region decorated by *E. coli* ssDNA-binding protein.

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 of t-circles (Fig. 1*C*).

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 ϕX174 DNA (25). The fractions from the gradient were subjected to Southern blot analysis with the TEL51 and TEL51C probes (Fig. 2*A*). Double-stranded telomeric DNA circles hybridizing to both probes were found within the lower part of the tube (Fig. 2*A*, *lanes 2* and *3*). Molecules with higher buoyant density contained mostly ssDNAs hybridizing to TEL51C (Fig. 2*A*, *lanes 6* and *7*). A small portion of the molecules in these fractions hybridized to both oligonucleotides, suggesting their double-stranded nature (Fig. 2*A*, *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. 2*A*), 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. 2*B*) 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 organello* DNA replication assays on purified *C. parapsilosis* mitochondria. After initiation of the reaction by addition of [α-^{32}P]dCTP, aliquots were taken at 0, 2, 5, 10, and 15 min, and DNA was isolated and digested with BglII, HindIII, PvuII, and XbaI endonucleases, allowing discrimination between telomeric and internal restriction enzyme fragments of the mtDNA (Fig. 3*A*). 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 BglII-

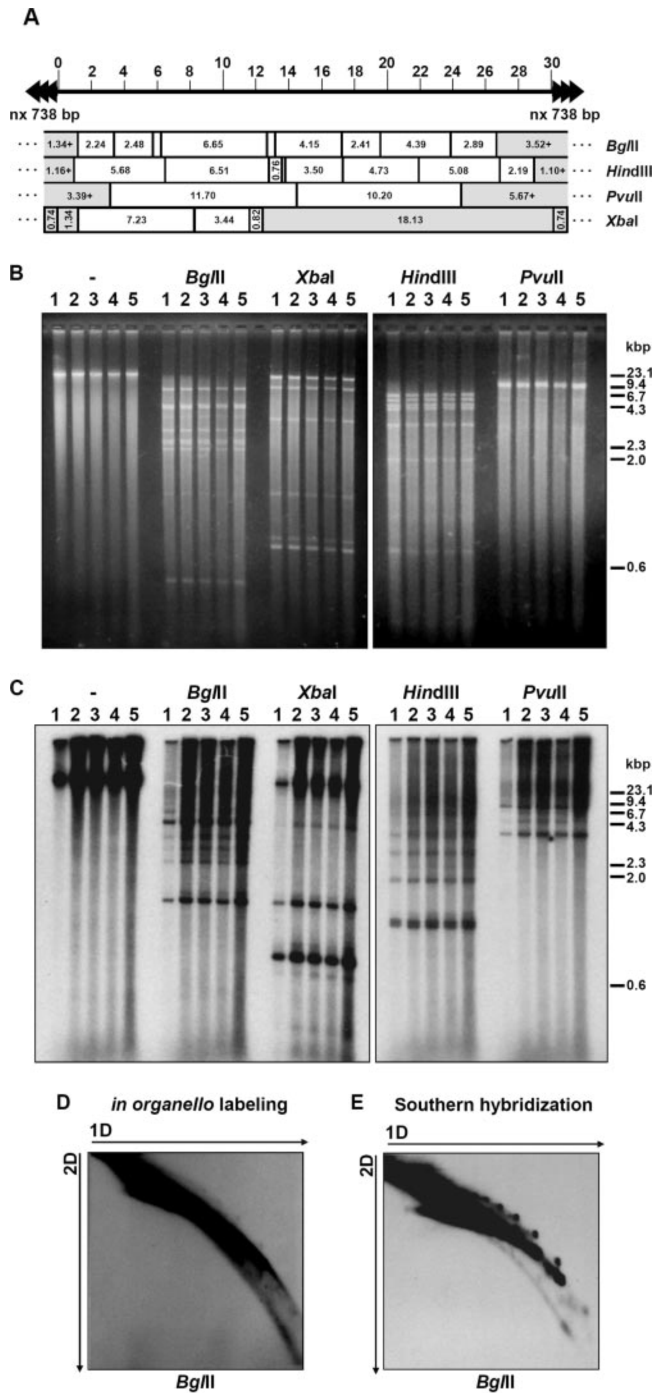
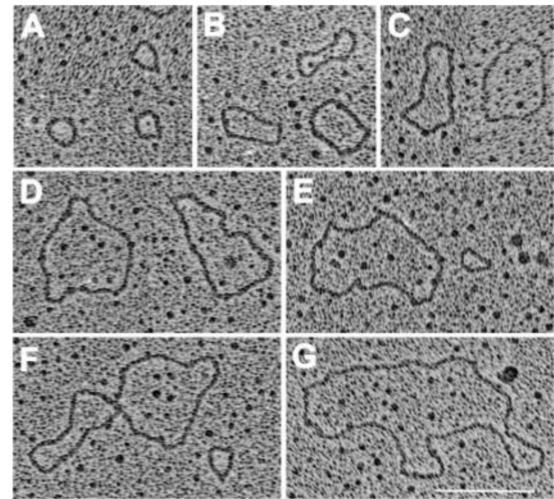


FIG. 3. **Telomeric regions of mtDNA of *C. parapsilosis* are hot spots of DNA synthesis.** *A*, the restriction enzyme map of the linear mtDNA of *C. parapsilosis* strain SR23 (30.922 + nx738 bp) (38). *Gray* areas represent preferentially labeled mtDNA fragments. *In organello* replication assays were performed as described under "Experimental Procedures." The reactions were stopped at the indicated time points, and mtDNA was isolated, digested with a corresponding restriction endonuclease, and subjected to agarose gel electrophoresis (*B*) followed by autoradiography (*C*). *D*, an aliquot of the BglII-digested mtDNA (*panels B* and *C*, *lanes 5*) was subjected to two-dimensional gel electrophoresis as described under "Experimental Procedures" to visualize the DNA species migrating above and below the main DNA population. *E*, the profile of DNA species on Southern blot of BglII-digested mtDNA probed with the 738-bp EcoRI-EcoRI fragment representing the telomeric unit is shown for comparison.

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. 3*D*). 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. 3*E*). 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.



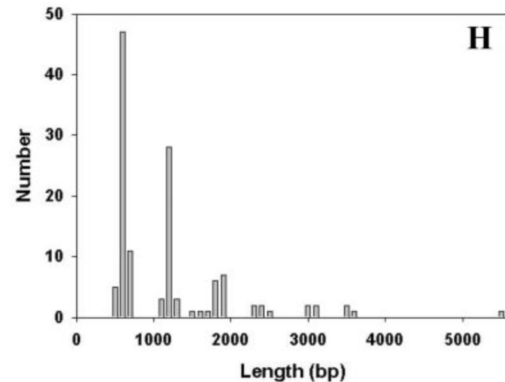


FIG. 4. **T-circles in a strain of *C. parapsilosis* with a variant telomeric repeat.** T-circles isolated from purified mitochondria of the strain MCO448 of *C. parapsilosis* by alkaline lysis were relaxed with DNase I as described in Ref. 12. Aliquots prepared for EM were directly adsorbed to thin carbon foils and rotary shadow-cast with tungsten. *Bar* represents 0.25 μm. Circles with contour length classes of 0.6 kb (*A*, *E*, *F*, *right*), 1.25 kb (*B*), 1.9 kb (*C*), 2.5 kb (*D*), 3.1 kb (*E*), 3.7 kb (*F*), and 5.6 kb (*G*) are shown. *H*, *histogram* of circle lengths measured from micrographs as in *panels A–G*.

A recent survey of several strains of *C. parapsilosis* revealed that an intraspecific 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).

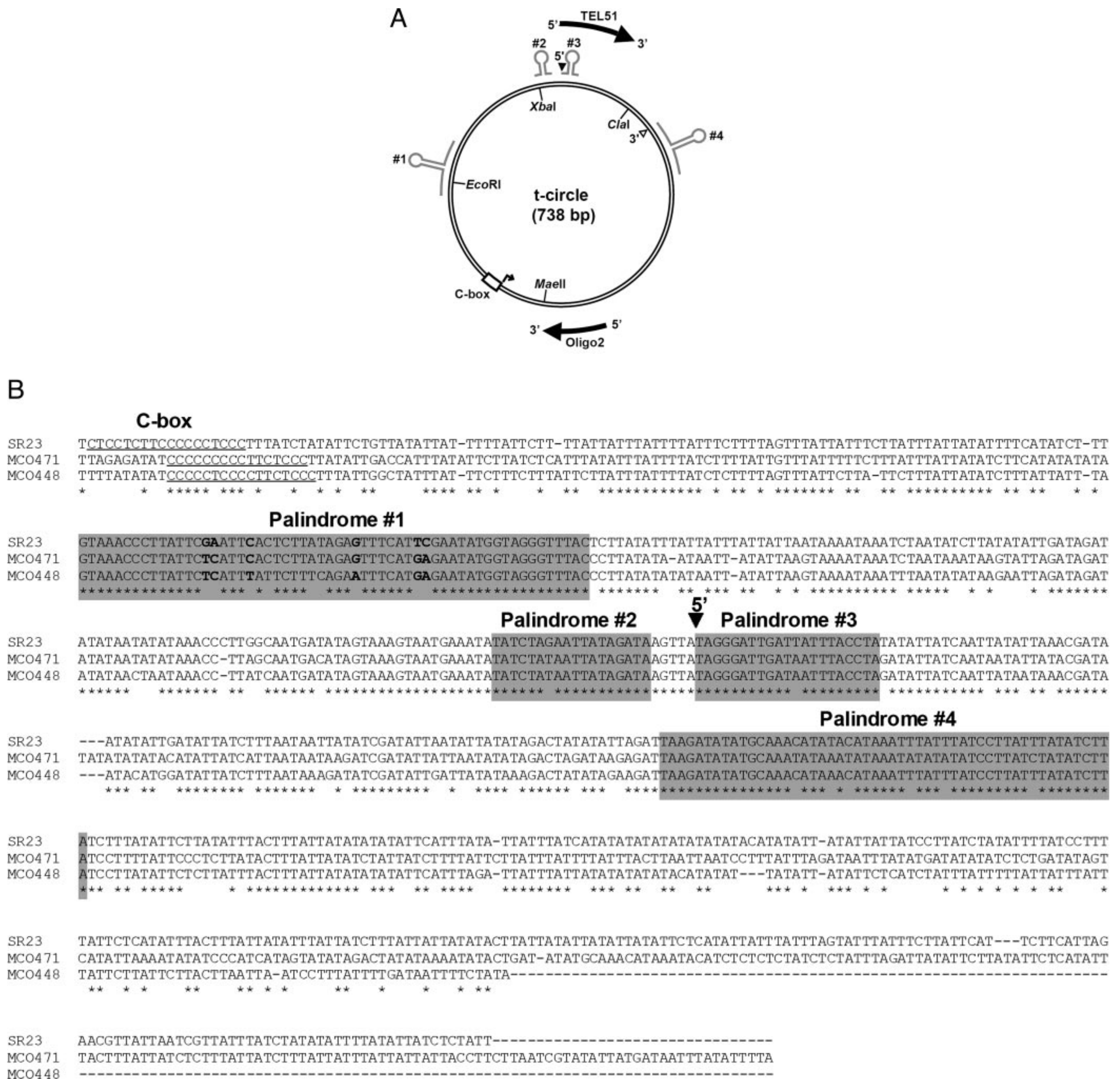


FIG. 5. **Putative regions implicated in the dynamics of t-circles of *C. parapsilosis.*** *A*, structure of the t-circle monomer (738 bp) from the *C. parapsilosis* strain SR23. The C-box and palindromes 1–4 are shown. *Filled* and *open triangles* at the base of palindromes 3 and 4 indicate positions corresponding to the 5′- and 3′-ends of the mitochondrial chromosome, respectively (see also Fig. 1*A*). *Bold arrows* illustrate regions corresponding to the oligonucleotides TEL51 and Oligo2. The *bent arrow* indicates the start of the telomeric unit within the array. *B*, DNA sequence alignment of variant t-circles isolated from mitochondria of *C. parapsilosis* strains SR23, MCO471, and MCO448. The C-box that may be involved in recombinational transactions is *underlined*. Note that subterminal 554-bp repeats terminate with a guanine-rich domain (G-box) (see Fig. 1*A*) that may be also involved in these events. Four conserved palindromic sequences are *shaded*. The position corresponding to the terminal nucleotides of the mitochondrial chromosome (16) at the base of palindrome 3 is labeled as 5′. Bases that covary in the stem of palindrome 1 are shown in *bold*.

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. 5*A*). 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. 1*A*) 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. 5*B*) 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 functions were originally mediated by telomeric loops (8, 23). As mitochondrial telomeres seem to fulfill analogous biological functions and display all the essential features of their nuclear counterparts, *i.e.* they consist of arrays of tandem repeats and possess a protruding single-stranded overhang (16) that is capped by specific telomere-binding protein (33, 34) and telomeric loops (27), mitochondria harboring linear mitochondrial genomes may represent a molecular fossil (35) of the original telomere replication strategy (36). Telomeric loops seem to represent a general, evolutionary conserved property of terminal tandem arrays and may explain several telomere-related phenomena such as capping function, masking the ends from DNA repair machinery providing a solution to the end-replication problem, and telomere rapid deletion (7, 8). However, their formation requires pre-existing and sufficiently long arrays of terminal repeats. Our results indicate that displacement mode of DNA synthesis at t-circles generates long tandem arrays of the telomeric sequence that may recombine with the linear DNA molecules to lengthen the termini (9). From the evolutionary point of view it is important to note that the rolling-circle replication strategy is common among various replicons in prokaryotic and eukaryotic organisms. Telomeres thus may have evolved from a selfish element functionally related to the t-circles that integrated into primitive eukaryotic, presumably circular, genome, forced its conversion toward a linear form, and produced amplified tandem arrays at its termini (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 primordial system of nuclear telomere maintenance.

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