Journal of Applied Biomedicine
2: 71–79, 2004
ISSN 1214-0287

REVIEW

The chromosome end replication: lessons from mitochondrial genetics

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Received 30th May 2003.
Published online 16th July 2003.

Summary
The widespread occurrence of linear mitochondrial genomes evokes intriguing questions concerning the evolutionary origin and mechanisms leading to the emergence and stabilization of linear DNA genophores. The study of their replication strategies opens a unique possibility of discovering alternative solutions to the end-replication problem and of elucidating how these mechanisms have appeared in evolution. The analysis of linear mitochondrial genomes in organisms belonging to different phylogenetic lines indicates that their evolutionary emergence was accompanied by the generation of various types of terminal structures, the adaptation of existing replication machinery and by the application of different strategies of the telomere replication. This scenario is illustrated by the molecular anatomy and replication of the linear mitochondrial genome in the opportunist yeast pathogen *Candida parapsilosis*. Recent studies have revealed the existence of extragenomic minicircular molecules derived from the telomere repeats that seem to participate in the novel pathway of telomere maintenance. Importantly, several lines of evidence indicate that a similar mechanism may also be involved in the alternative, telomerase-independent, maintenance of nuclear telomeres in higher eukaryotes, including human telomerase-negative tumor cells.

Keywords: end-replication problem – linear mitochondrial DNA – telomere – replication – evolution

AN INTRODUCTION TO THE CHROMOSOME END-GAME

The end-replication problem of linear DNA molecules was formulated in the early 1970s (Olovnikov 1971, Olovnikov 1973, Watson 1972) and its principle is based on the observations that (i) all known DNA polymerases are able to synthesize a new complementary polynucleotide strand only in the 5' to 3' direction, and (ii) to initiate DNA synthesis, conventional DNA polymerases require an RNA primer that is subsequently removed. Watson and Olovnikov independently recognized that without a special compensatory mechanism the application of these basic rules to the synthesis of linear DNA would result in a loss of sequences at the 5' end of the molecules (Fig. 1A). Furthermore, Olovnikov in his theory of marginotomy postulated that the erosion of terminal sequences in subsequent rounds of DNA replication plays the role of the biological clock in the processes of cellular senescence and aging (Olovnikov 1973). To solve the end-replication problem and thus to circumvent the fatal consequences of incomplete replication the eukaryotic cells possess special devices at the end of the chromosomes called telomeres. The telomeric sequences of most of the eukaryotic organisms consist of long arrays of short (5–8 bp) tandem repeat units rich in guanine nucleotides (reviewed in Biessmann and Mason 1994). Their replication is ensured by a special ribonucleoprotein enzyme –

telomerase. This enzyme was originally discovered in macronuclei of the ciliate *Tetrahymena* and later identified in nuclei of yeast, plant and animal cells (Cohn and Blackburn 1995, Fajkus et al. 1996, Greider and Blackburn 1985, Greider and Blackburn 1987, Kim et al. 1994). An essential component of telomerase is the RNA molecule containing a short sequence domain complementary to a telomeric repeat motif. By reverse transcription of this domain, telomerase adds repetitive units to the 3' end of chromosomal DNA and thus compensates for the loss of terminal sequences encountered during replication (Fig. 1B). The activity of telomerase *in vivo* is regulated by various factors and the number of telomeric repeats is determined by an equilibrium between the processes increasing and decreasing the length of telomeres (for review see McEachern et al. 2000).

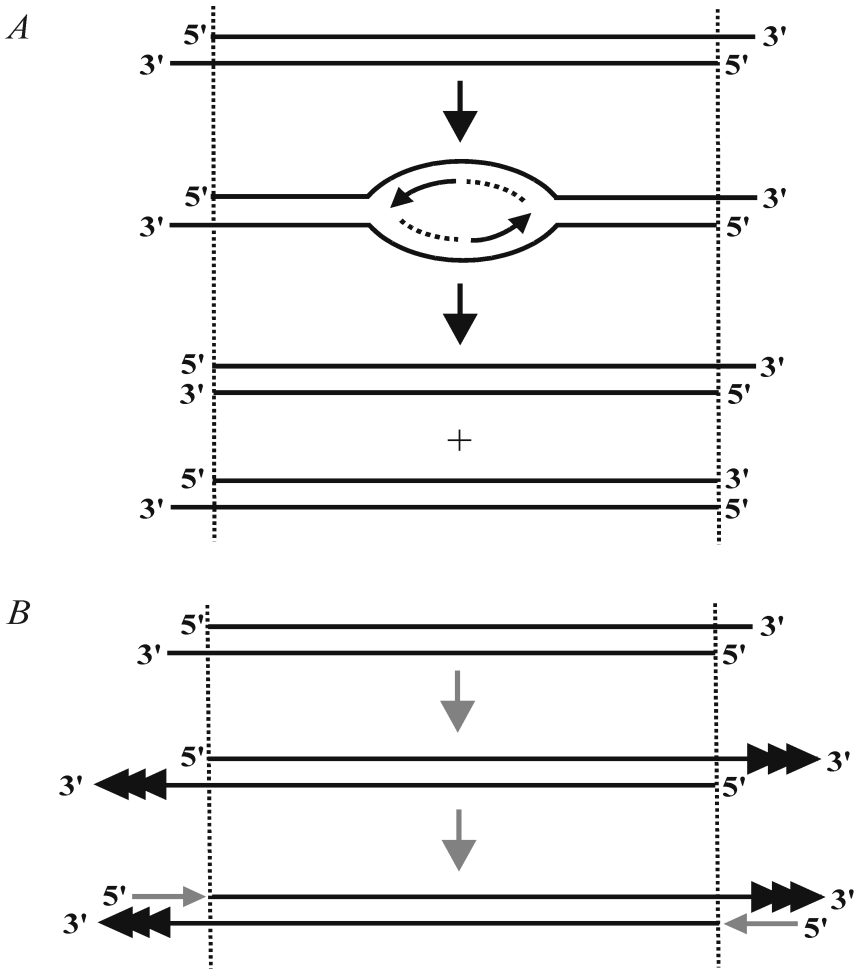

Fig. 1. The end-replication problem. (*A*) A standard DNA replication machinery is unable to fill in gaps at the 5' ends of linear molecules. (*B*) The telomerase solves this problem by an extension of the 3' strand by arrays of telomeric tandem repeats. This allows the synthesis of the complementary strand by a conventional DNA polymerase

In human somatic cells the telomerase activity is down-regulated as the consequence of cell differentiation, the telomere length of their chromosomes decreases with the number of cell divisions and subsequently these cells enter the senescence phase. In contrast, embryonic cells, blood stem cells, immortal cell lines and many tumor cells exhibit the telomerase activity that maintains the length of their chromosomes. These observations have resulted in the formulation of the telomere hypothesis suggesting a key role of telomere dynamics in the process of tumorigenesis. According to this hypothesis, telomere shortening is considered as a tumor suppression mechanism and the re-activation of telomerase is an essential step for the origin and infinite growth of immortal cell lines (reviewed in Greider and Blackburn 1996, Shay et al. 2001). Based on this hypothesis the telomerase is not only a useful diagnostic marker but also represents a promising target for anti-cancer therapy. Therefore the telomerase inhibitors might become a reasonable and effective therapeutic tool (Shay and Wright 2002).

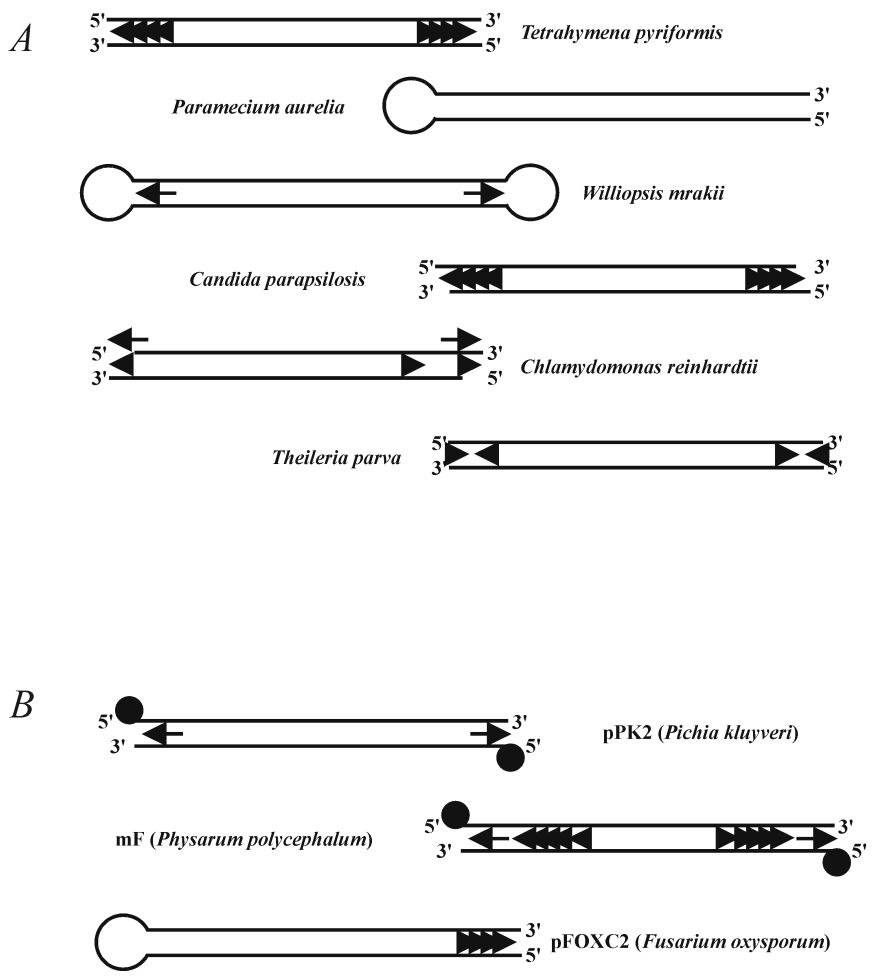

Fig. 2. Variability of mitochondrial telomeric structures of linear mitochondrial genomes (*A*) and linear mitochondrial plasmids (*B*). These linear genophores terminate by (i) a covalently-closed single-stranded hairpin loop at one (*Paramecium aurelia,* pFOXC2) or both ends (*Williopsis mrakii*), (ii) arrays of tandem repetitions (*Tetrahymena pyriformis*, *Candida parapsilosis*), (iii) inverted terminal repeats with a long 3' single-stranded non-complementary extentions (*Chlamydomonas reinhardtii*), (iv) complex repetitions (*Theileria parva*, mF plasmid), and terminal proteins covalently attached to 5' ends of DNA molecules (plasmids mF, pPK2)

However, the situation seems to be far more complicated than is described in the simple model above and the telomere hypothesis has recently been revisited (Autexier and Greider 1996). Results from many laboratories indicate that about 10% of human immortal cell lines do not exhibit detectable telomerase activity. It has been demonstrated that certain telomerase-negative human tumors and tumor-derived immortal cell lines acquired very long telomeric blocks through unknown telomere-lengthening mechanism(s). The maintenance of telomeres by alternative, telomerase-independent, mechanisms might be important in certain types of cancer, and in therapeutic attempts, the emergence of such mechanisms may represent a potential source of resistance of tumor cells to telomerase inhibitors (Dunham et al. 2000, Reddel 2003, Reddel et al. 2001).

Alternative mechanisms (ALT) may run in parallel and/or, more importantly, they may emerge in telomerase-deficient or telomerase-inhibited cells to enable the chromosome elongation and thus tumor progression. The emergence may be due to mutations or to a mobilization, by tinkering assembly, of potentially available modules, originally destined to other functions (Jacob 1977). It is therefore of importance to understand the nature of both telomerase-dependent and –independent mechanisms of the telomere elongation. Such research may provide the basis for development of combined treatment based on the inhibition of all telomere maintenance pathways.

The ALT mechanisms represent primary pathways in several organisms such as fruit flies, mosquitoes and some plants. Two molecular events were shown to be responsible for ALT pathways: homologous recombination and transposition (Biessmann and Mason 1997, Dunham et al. 2000). However, additional solutions to the problem may exist.

The end-replication problem is not limited to the nuclear eukaryotic chromosomes, but it is virtually related to the replication of any genophore that exists *in vivo* as a linear DNA molecule (e.g. chromosomes of several bacteria, viruses, plasmids). The study of their replication strategies may be of great value from the aspect of alternative modes of telomere replication. Evolutionary distant linear genophores have evolved several independent strategies that allow the complete synthesis of linear DNA. This is exemplified by the range of solutions to the end replication problem observed for diverse linear genophores: (i) the re-circularization of the DNA (bacteriophage λ); (ii) the dimerization of incompletely replicated DNA molecules through their terminal complementary sequences (bacteriophage T7); (iii) the Cavalier-Smith-Bateman scheme in the case of molecules with a terminal covalently-closed hairpin loop (poxviruses, linear chromosomes of *Borrelia*), and (iv) the priming of DNA replication by a terminal protein covalently attached to a 5' end of the molecule (adenoviruses, linear plasmids, linear chromosomes of *Streptomyces*). The studies of replication strategies employed by different linear DNA genophores may disclose additional molecular principles of alternative solutions to the end-replication problem and elucidate how these, apparently independent, pathways appeared in evolution.

LINEAR MITOCHONDRIAL GENOMES IN YEASTS AS THE MODEL SYSTEM

In contrast to a general belief that mtDNA is a circular molecule, mitochondrial genomes in many organisms are represented by linear DNA molecules with specific telomeric structures (reviewed in Nosek et al. 1998). The organisms harboring linear mitochondrial DNA of defined length belong to wide variety of taxa (e.g. protozoa, algae, fungi, and even several multicellular organisms). Also these linear genomes had to evolve special terminal structures that ensure their complete replication. Mitochondrial telomeres exhibit a surprising diversity of DNA sequences and molecular forms (Fig. 2A,B) indicating that the evolution of linear mitochondrial genomes was accompanied by the generation of various types of terminal structures and hence by the application different strategies of the telomere replication (for more detailed review see Nosek and Tomaska 2002). Linear mitochondrial genomes thus provide a unique system for examining how these genophores found different solutions to the end-replication problem.

In several cases mitochondrial genomes set up a similar solution to the linear genophores of viruses, plasmids and bacterial chromosomes. This may suggest either a horizontal transfer of pre-existing telomere modules from another linear genophore or an independent successful attempt to find the right strategy for maintenance of the telomere.

An inspection of mtDNA in non-conventional yeast species uncovered an unexpectedly high occurrence of the linear form (Fukuhara et al. 1993). Linear mitochondrial genomes, identified in yeast species belonging to genera *Williopsis* and *Pichia*, possess at both ends long inverted repeats with a covalently closed single-stranded hairpin, reminiscent of the poxvirus telomeres. Replication of this type of linear mtDNA seems to proceed through monomeric and dimeric circular molecules with mutual interconversion of linear and circular forms (Dinouel et al. 1993, Fukuhara et al. 1993).

Although it was originally thought that all yeast linear mtDNAs have similar telomeres, an analysis of mtDNA in *Candida parapsilosis*, *Candida salmanticensis* and *Pichia philodendri* revealed a novel type of terminal structure and the corresponding linear genome was designated as type II linear mtDNA (Nosek et al. 1995). Mitochondrial telomeres of the yeast *C. parapsilosis* consist of inverted terminal repeats themselves consisting of arrays of tandem repetitions a 738 bp unit. The number of repetitive units varies thus giving rise to a population of molecules heterogeneous in size. The last incomplete unit terminates with a 5' single-stranded overhang of about 110 nucleotides (Nosek et al. 1995). Such a unique structure of mitochondrial telomere evokes questions about how the terminal 5' single-stranded extensions are generated and stabilized in mitochondria and why DNA polymerase stops at the 3' end and does not fill the protruding overhang. Since the shortest molecules lack a complete repeat unit they would be unable to restore the missing sequence without a special mechanism and might be preferentially lost at replication. Considering the telomeric structure, the replication strategy of type II linear mtDNA probably significantly differs from that of type I linear mtDNA.

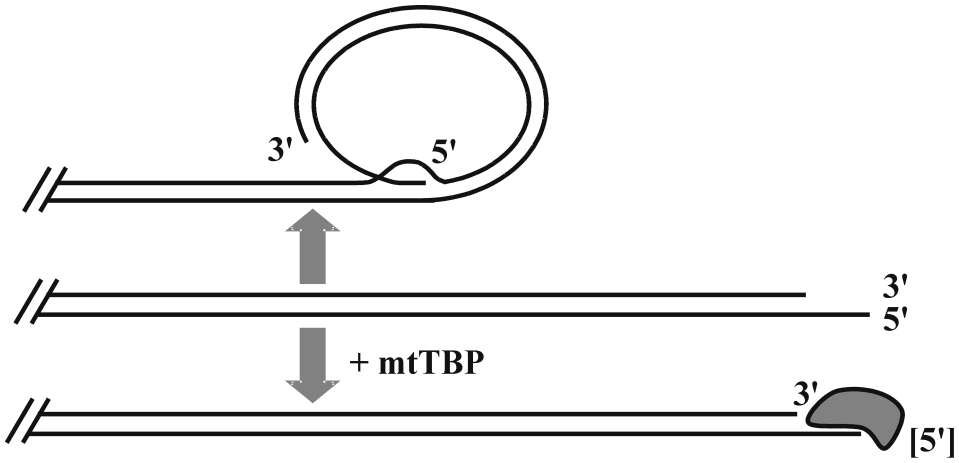

Fig. 3. Telomeric loops (t-loops) at the ends of linear mtDNA of *C. parapsilosis*. Mitochondrial t-loops are presumably formed by an invasion of the 5' overhang to the double-stranded region of the telomere. The mtTBP may compete with the t-loop formation. Hence, both mtTBP and t-loops may be involved in the capping function of the mitochondrial telomere

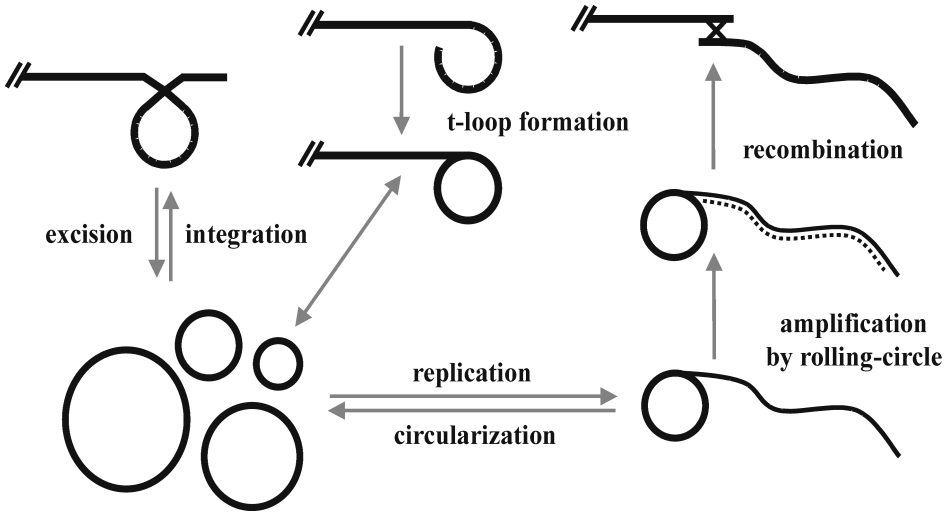

Fig. 4. An involvement of extragenomic telomeric minicircles and t-loops in the novel telomerase-independent pathway. An excision/integration of minicircles may result in the shortening/lengthening of telomeres. Minicircles are amplified *via* rolling-circle mechanism generating linear products that recombine with mtDNA to extend the telomeres. In addition, it is presumed that t-loops are involved in the telomere dynamics and the minicircle formation

THE TELOMERE ARCHITECTURE: AN INSIGHT INTO THE BIOLOGIAL ROLE OF MITOCHONDRIAL TELOMERES

To establish the biological roles of mitochondrial telomeres we focused our research on linear mitochondrial DNA (mtDNA) of the pathogenic yeast *C. parapsilosis*. Since the organization of mitochondrial telomeres of *C. parapsilosis* remotely resembles telomeres of eukaryotic chromosomes a similar mechanism for their elongation and stabilization might be presumed. Although the idea of the existence of a mitochondrial counterpart of telomerase is highly attractive, it has not been substantiated in any convincing way. Rather telomerase-independent replication mechanisms (*e.g.* non-reciprocal homologous recombination and transposition) could be envisaged for linear mitochondrial genomes of this type. Even though the biological role of mitochondrial telomeres is yet not fully understood, by analogy with eukaryotic nuclear telomeres it is possible to postulate their significance for the complete replication of the linear genophore and its protection against degradation by nucleases.

The analysis of proteins specifically interacting with terminal structures of linear mitochondrial DNA and mediating telomere functions offers a unique possibility for uncovering the role of mitochondrial telomeres in the replication and

maintenance of linear mtDNA and the evolutionary constraints that led to their emergence. Using the gel retardation and UV-crosslinking assays we identified a protein mtTBP (mitochondrial telomere-binding protein) that specifically recognizes a terminal structure of mtDNA of *C. parapsilosis*. The protein was purified to homogeneity using a DNA-affinity chromatography. In its native state, mtTBP forms homo-tetramers and protects the 5' single-stranded telomeric overhang against various DNA modifying enzymes (Tomaska et al. 1997). A nuclear gene encoding mtTBP was isolated and sequenced. *In silico* analysis revealed that mtTBP displays significant homology with a family of bacterial and mitochondrial single-stranded DNA-binding (SSB) proteins. This suggests that mtTBP might have evolved from non-specific mitochondrial SSB protein. However, in contrast to other SSB proteins that bind with high affinity to single-stranded DNA without apparent sequence specificity, mtTBP was adapted to recognize the ends of linear mtDNA and to protect a sequence of the 5' single-stranded overhang of mitochondrial telomere from enzymatic degradation (Nosek et al. 1999, Tomaska et al. 2001).

Based on the nature of mtTBP, a hypothesis was proposed that evolutionary emergence of linear mtDNA was accompanied by (i) the generation of various types of terminal structures and (ii) the adaptation of the component(s) of mtDNA replication machinery. This means that the cell with an accidentally linearized mitochondrial genome may mobilize a pre-existing set of proteins to solve the problem of complete DNA replication. Further functional studies of mtTBP and other mitochondrial telomere-binding proteins may provide an insight into mechanisms that enabled the fortuitous evolutionary emergence of linear mitochondrial genomes. Recently, the electron microscopic analysis of psoralen-treated mitochondria of *C. parapsilosis* revealed a small, but reproducible fraction (~5%) of linear mtDNA molecules possessing a telomeric loop structure (t-loop; Tomaska et al. 2002).

Table 1. **Extrachromosomal telomeric repeats** (ECTRs)*

Animals	
Xenopus laevis	telomeric extrachromosomal circular DNA (tel-eccDNA)
rodent and human cell lines	telomeric small polydisperse circular DNA (tel-spcDNA)
mouse Atm $^{-/-}$ cells (telomerase-positive)	ECTR
human Atm $^{-/-}$ cells (telomerase-positive)	ECTR
human telomerase-negative cell lines	linear ECTR
Plants	
wheat	nuclear extrachromosomal DNA
Yeasts	
Saccharomyces cerevisiae	subtelomeric Y' circles
Kluyveromyces lactis	synthetic DNA circles with telomeric array
Yeast mitochondria	
Candida parapsilosis	extragenomic telomeric minicircles (TMC)
Candida salmanticensis	extragenomic telomeric minicircles (TMC)
Pichia philodendri	extragenomic telomeric minicircles (TMC)

* The table is summarized from Bucholc and Buchowicz 1995, Cohen and Lavi 1996, Cohen and Mechali 2002, Cohen et al. 1997, Hande et al. 2001, Horowitz and Haber 1985, Louis and Haber 1990, Natarajan and McEachern 2002, Nosek and Tomaska 2002, Ogino et al. 1998, Regev et al. 1998, Tokutake et al. 1998, Tomaska et al. 2000

In contrast to the t-loops found at the tips of mammalian chromosomes (Griffith et al. 1999), the mitochondrial counterpart is presumably formed by an invasion of a 5' strand into the double-stranded region of the mitochondrial telomere. Hence, mitochondrial t-loops do not provide an alternative solution for the end-replication problem. However, t-loops may function in concert with mtTBP to protect the ends of linear mtDNA and thus take part in a capping function of mitochondrial telomeres (Fig. 3).

TELOMERE MINICIRCLES: KEY PLAYERS IN THE NOVEL TELOMERASE-INDEPENDENT PATHWAY

Investigations by two-dimensional (2D) neutral/neutral agarose gel electrophoresis, DNA-DNA hybridization as well as by electron microscopy, have uncovered the extragenomic minicircular double-stranded DNA molecules composed solely of a mitochondrial telomeric sequence (Tomaska et al. 2000). Their lengths correspond to integral multimers of the tandem repeat unit (*i.e.*, n x738 bp). Moreover, the telomeric minicircles are not limited to *C. parapsilosis* but they have also been identified in two other yeast species, *C. salmanticensis* and *P. philodendri*, suggesting that their occurrence is more general. Our further experiments indicate that the telomeric minicircles replicate autonomously within mitochondria via a rolling-circle replication strategy (*unpublished results*). This mechanism generates long linear arrays of telomere repeats that are presumed to recombine with linear mtDNA thus preventing the terminal shortening of molecules.

Based on the molecular anatomy of mitochondrial telomeres in *C. parapsilosis* we proposed a hypothesis suggesting that extragenomic telomeric minicircles and t-loops participate in a novel pathway of telomere maintenance (Fig. 4). Moreover, an active role of telomeric minicircles in yeast mitochondria may parallel alternative mechanisms of nuclear telomere maintenance in the presence of telomeric small polydisperse circular DNAs (tel-spcDNA) found in several telomerase-negative tumor cell lines (Regev et al. 1998) and *Xenopus* oocytes (Cohen and Mechali 2002) or an elongation of telomeres by recombination with DNA circles demonstrated in *Kluyveromyces lactis* (Natarajan and McEachern 2002) (Table 1).

Taken together, studies on mitochondrial telomeres go beyond the field of mitochondrial genetics and may shed some light on the nature and evolution of alternative, telomerase-independent, solutions to the end-replication problem that has recently intensified interest in telomere biology.

ACKNOWLEDGEMENTS

We wish to thank the members of our laboratories for discussions and L. Kováč (Department of Biochemistry, Comenius University, Bratislava) for helpful comments and continuous support. The work in our laboratory is supported by grants from the Howard Hughes Medical Institute (55000327), the Slovak grant agencies VEGA (1/9153/02 and 1/0006/03) and APVT (20-003902), and the Fogarty International Research Collaboration Award (1-R03-TW05654-01). J.N. was supported by a fellowship from the EMBO (ASTF 153.00-02).

Simultaneously published in J. Berger (ed): Advances in Cell Biology. Kopp Publ., České Budějovice 2003, pp.61–78.

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