



Yeast telomeres: how to ignore essential double-strand DNA breaks?

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Summary

DNA looping is one of the mechanisms involved in telomere maintenance. It probably provides a solution not only to 'the end-replication problem', but also for the protection of chromosomal ends against degradation enzymes and, as typical double-strand breaks, from DNA repair machinery. Telomeric loops (t-loops) formed by an invasion of protruding 3' overhangs into the double-stranded telomeric regions were observed in a variety of organisms ranging from ciliates to mammals. Genetic data indicate that looping also occurs at the telomeres of *Saccharomyces cerevisiae*, suggesting its importance for telomere function in yeast. However, several observations argue against the presence of 'true' t-loops in the budding yeast telomeres (e.g. the lack of TRF-like protein, heterogeneous telomeric sequences). Instead, telomeres in *S. cerevisiae* appear to form fold-back structures mediated by protein-protein interactions. To directly visualize the telomeric structure in budding yeast, we developed a system based on a mini-chromosome carrying an array of *lac* operator sequences allowing its purification by the *lac* repressor affinity column. In contrast to budding yeast, the fission yeast *Schizosaccharomyces pombe* contains a homologue of the human telomeric protein TRF2, designated Taz1p. As the TRF2 protein has been implicated in remodelling telomeres into t-loops, the ability of Taz1p to promote t-loop formation is examined by electron microscopy using purified protein and synthetic templates containing a double-stranded fission yeast telomeric tract. Our studies should shed some light not only on telomeric architecture in yeast, but should also be instrumental in deciphering detailed telomeric structure in higher eukaryotes.

Keywords: telomere – t-loop – Taz1 – fission yeast – budding yeast – chromatin – silencing

INTRODUCTION

Telomeres, the DNA-protein complexes at the ends of linear chromosomes, stabilize the termini and protect them from end-to-end fusion. With a few exceptions, telomeres of most eukaryotic cells consist of an array of short repeats rich in guanines on the strand running from 5' to 3' towards the chromosome end. Another general feature of nuclear telomeres is the presence of a 3' overhang of the G-rich strand at the termini (McEachern et al. 2000).

The exciting studies on the replication of eukaryotic chromosome ends resulted in the formulation of the 'telomere hypothesis', suggesting a key role for telomere dynamics in the process of tumorigenesis (Fig. 1). According to this hypothesis, telomere shortening accompanied by decapping is considered a tumor suppression mechanism and the re-activation of telomerase – a specialized nucleoprotein enzyme that extends the chromosome tips, thus compensating the losses of terminal sequences encountered during replication – is an essential step in the origin and infinite growth

of immortal cell lines (Bodnar et al. 1998, Greider 1999). Based on this scenario, the telomerase represents a promising target for anti-cancer therapy and the telomerase inhibitors might become a reasonable and effective therapeutic tool (Hahn et al. 1999, Raymond et al. 1996). However, the situation seems to be far more complicated and the telomere hypothesis has recently been revisited (Autexier and Greider 1996, Holt et al. 1996). It has been demonstrated that telomerase-negative human tumors and tumor-derived immortal cell lines, acquired very long telomeric blocks through

unknown telomere-lengthening mechanisms. 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 toward the telomerase inhibitors (Biessmann and Mason 1997, Bryan et al. 1997, Bryan et al. 1995, Bryan et al. 1997, Bryan and Reddel 1997, Colgin and Reddel 1999, Raymond et al. 1996, Reddel et al. 2001, Reddel et al. 1997).

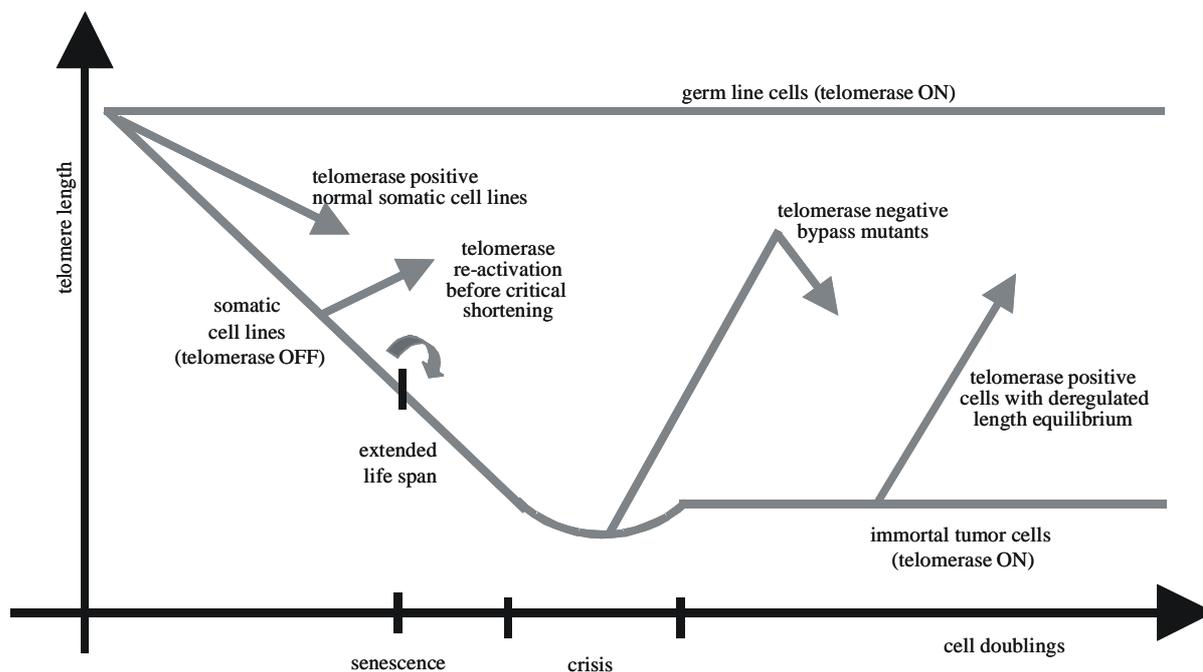


Fig. 1. **The telomere/telomerase hypothesis** (according to Autexier and Greider 1996).

Although telomerase-independent mechanisms of telomere elongation in human immortal cells are well documented, their nature is not yet understood. The analysis of telomerase-independent mechanisms in lower eukaryotes has resulted in several important observations. For example, *S. cerevisiae* mutants with defects in the telomerase component revealed that yeasts reconstitute chromosomal ends by a *RAD52*-dependent recombination mechanism (Wang and Zakian 1990). By analogy, a recombinational mechanism was proposed also for mouse cells with a knocked-out gene for the telomerase component (Blasco et al. 1997, Lansdorp, 1997). Recently, Reddel and co-workers identified a *RAD52*-dependent recombination as the major pathway for restoring telomeres in telomerase-negative cells (Dunham et al. 2000).

However, recombination might not be the only way of survival. *Schizosaccharomyces pombe*

mutants in the gene coding for telomerase RNA solve the problem either by a recombinational *RAD52*-dependent mechanism or by circularization of their chromosomes (Naito et al. 1998, Nakamura et al. 1998). Moreover, rare survivors of *Kluyveromyces lactis* with the double mutation $\Delta ter1 \Delta rad52$ might suggest the existence of yet another mechanism (McEachern and Blackburn 1996). In several organisms that include insect (*Chironomus*, *Anopheles*, *Drosophila*) and plant (*Allium*) species, a telomerase-independent strategy of telomere elongation naturally operates as a primary mechanism (Biessmann and Mason 1997). Although in many cases the unequal recombinational mechanism was proposed, *Drosophila melanogaster* maintains the ends of chromosomes by the repeated transfer of telomere-associated retrotransposons (Levis et al. 1993, Pardue et al. 1996, Pardue et al. 1997).

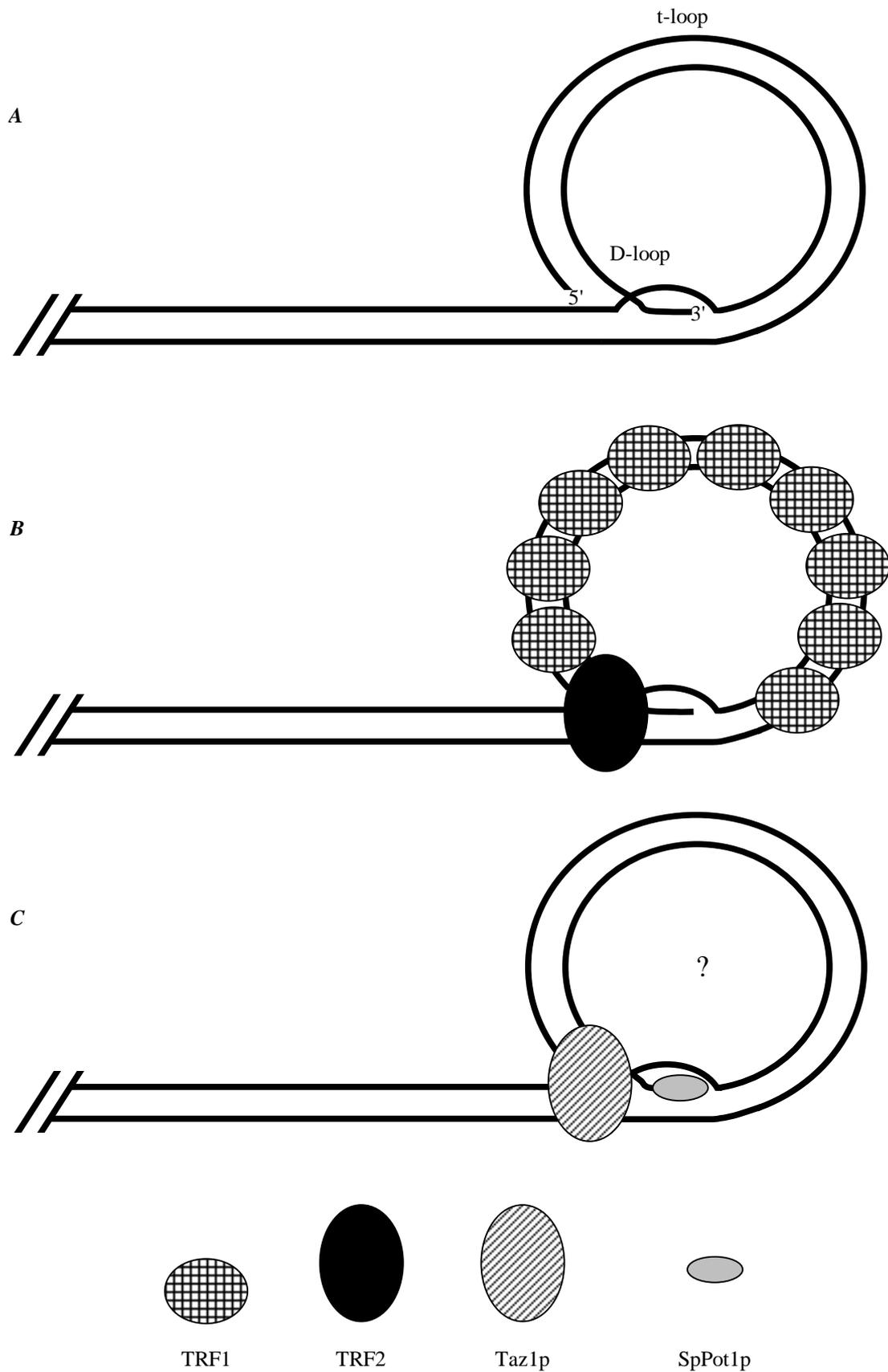


Fig. 2. A putative structure of capped telomere based on a t-loop. (Griffith et al., 1999) (A) Architecture of the t-loop. 3' single-stranded overhang invades a double-stranded telomeric region forming a D-loop. (B) Position TRF1 and TRF2 within the mammalian t-loop. Additional telomeric proteins, like Pot1p, Rap1p, Est1p, Rad50p, Mre11p, Nbs1p, Ku, and tankyrase, are not shown. (C) Hypothetical t-loop at fission yeast telomere.

TELOMERES – IGNORED DOUBLE-STRAND BREAKS

Telomeres exist in at least two different states (Blackburn 2000). The ‘open’ complex is probably needed for the telomerase to access the end of the telomeric DNA. The ‘closed’ complex caps and protects chromosome ends not only against degradation by nucleases, but importantly, against protein complexes mediating repair of double-strand breaks. In fact, loss of telomere capping seems to be one of the reasons for chromosomal end-to-end fusions and genomic instability (Desmazes et al. 2003). The changes in telomere architecture mediated by specific protein components of the telomeric chromatin are essential for proper functioning of the chromosomal ends (Rhodes et al. 2002).

The ‘closed’ state of the telomere is exemplified by the telomeric loops (t-loops) formed by an invasion of the 3' single-stranded (ss) overhang of mammalian telomeres into the duplex telomeric region (Griffith et al. 1999) (Fig. 2A). T-loops were subsequently found at the termini of micronuclear chromosomes of *Oxytricha nova* (Murti and Prescott 1999), at the telomeres of *Trypanosoma brucei* (Munoz-Jordan et al. 2001), as well as at the ends of linear mitochondrial DNA of the yeast *Candida parapsilosis* (Tomaska et al. 2002). It was suggested that t-loops would effectively hide the natural end of the chromosome to protect it from the DNA repair machinery. In addition, the 3' overhang mediated priming of the new DNA synthesis, followed by appropriate cleavages and gap filling, could give rise to a large increase in telomere size. In the original study on mammalian t-loops it was demonstrated that the formation of loops is mediated by a protein TRF2 (Griffith et al. 1999). TRF2 and its homologue TRF1 were identified as proteins binding to double-stranded (ds) telomeric DNA in the form of homodimers (Bilaud et al. 1997, Broccoli et al. 1997, Chong et al. 1995). Both TRF1 and TRF2 function to negatively regulate telomere length, as overexpression of either protein leads to the progressive shortening of the telomeric tract over many cellular divisions (Smogorzewska et al. 2000, van Steensel and de Lange 1997). Although TRF1 and TRF2 exhibit a high degree of homology, their role in telomere dynamics seems to be different, probably due to the differences at their N-termini (Karlseder 2003, Karlseder et al. 2002) (Fig. 2B). In contrast to TRF2, TRF1 is unable to promote t-

loop formation *in vitro*. Whereas TRF1 forms filamentous structures on telomeric repeat arrays and promotes parallel pairing of telomeric tracts *in vitro* (Griffith et al. 1998), TRF2 induces t-loop formation and binds preferentially at the ss/ds junction at the 3' telomeric overhang (Stansel et al. 2001). A 5'-TTAGGG-3' overhang of at least six nucleotides is required for the loop formation. Termini with 5' overhangs or blunt ends were deficient in the loop formation. On the other hand, the addition of non-telomeric sequences to the distal portion of a 3' overhang beginning with TTAGGG repeats, only modestly diminishes looping (Stansel et al., 2001).

Several nuclear proteins including Tin2 (Kim et al. 1999), tankyrase (Smith et al. 1998), and hRap1 (Li et al. 2000) associate with telomeric DNA via binding to TRF1 and TRF2 and thus modulate their activities including the t-loop formation. Other proteins, not directly associated with TRF1 and TRF2, may affect the t-loop dynamics. For example, Pot1 protein binds with a high affinity to the telomeric 3' single-stranded overhang (Baumann and Cech 2001) and can play a role in both recruiting telomerase to the chromosomal ends (Evans and Lundblad 1999) and affecting the ability of 3' telomeric overhang to invade the double-stranded region of the telomere. In addition, protein components of the nonhomologous end-joining pathway (Ku, Mre11, Nbs1, Xrs2 and Rad50) are essential constituents of the telosome (Chan and Blackburn 2002). This leads to a paradox: telomeres, typical double-strand DNA breaks, are kept intact partly due to the presence of proteins essential for a repair of the same type of DNA structures within internal parts of the genome. At the telomere, these proteins seem to target the chromosome ends for the action of telomerase as opposed to non-homologous end-joining. One possible solution to this apparent contradiction is that even though telomeres are double-strand DNA breaks, their higher-order architecture makes them resistant to the DNA repair machinery. Telomeric loops are obvious candidates for such structural design. If telomere looping provides a solution to the above problem, then it should be a general feature of chromosomal ends, including those in lower eukaryotes, like yeasts. The aim of our study is to test this hypothesis in both the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*.

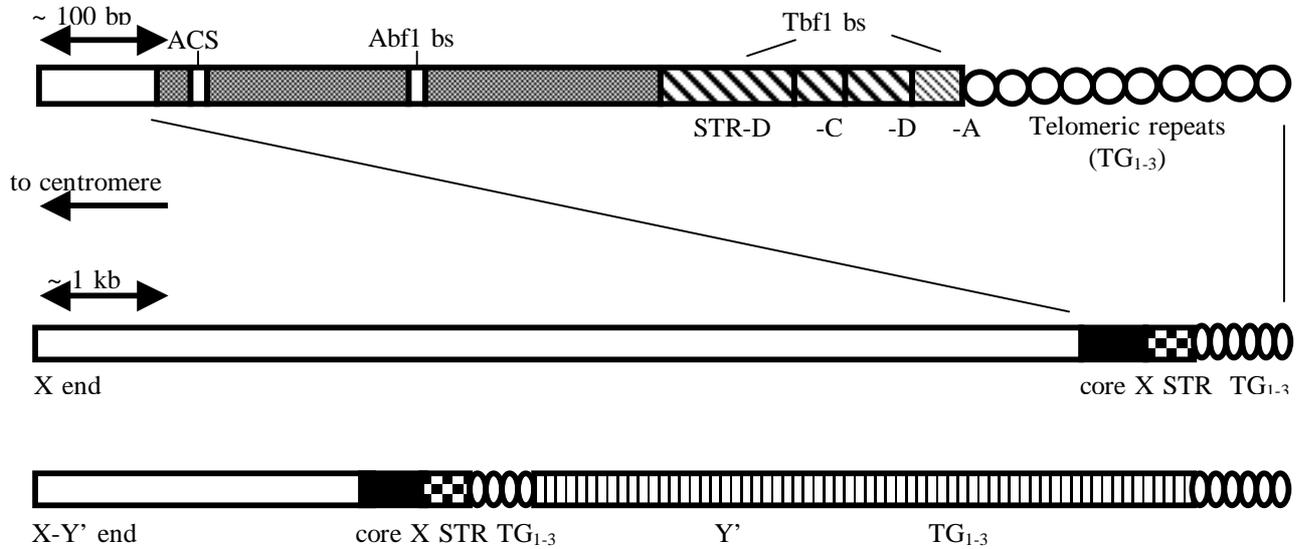


Fig. 3. **General structure of budding yeast telomeres** (adapted from http://www.le.ac.uk/ge/ejl12/research/telostruc/X_Y_description.html). Shown are both X end (containing only X element) and X-Y' end (containing one X and one to four Y' elements). All 32 chromosomal ends in *S. cerevisiae* contain an X element (composed of a core X element and several subterminal repeats (STR), whose detailed topology is shown in the upper part of the figure). Binding sites (bs) for Abf1p and Tbf1p are indicated.

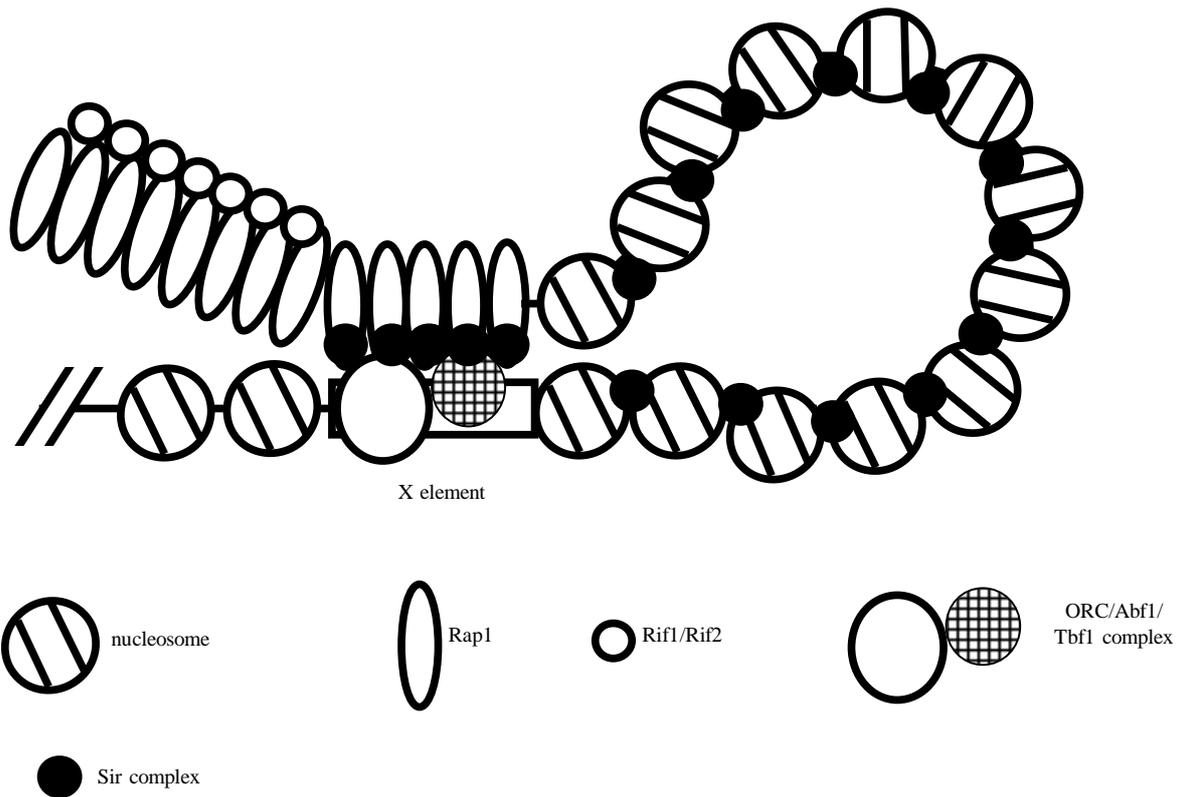


Fig. 4. **A model of looped telomeric chromatin in *Saccharomyces cerevisiae*** (according to Pryde and Louis 1999; see text for more details). For simplicity, only a subset of telomeric proteins is shown.

LOOPING AS A COMMON THEME IN TELOMERE ARCHITECTURE I: THE BUDDING YEAST

S. cerevisiae with its powerful genetic and molecular biology tools seems to be a strong candidate for detailed analysis of t-loop formation. However, it seems unlikely that the budding yeast telomeres form 'true' t-loops. For example, telomeric sequences in *S. cerevisiae* are very heterogeneous ($C_{2-3}ACA_{1-6}/T_{1-6}GTG_{2-3}$) (Shampay et al. 1984, Wang and Zakian 1990), decreasing the probability of invasion of the 3' telomeric overhang into the double-stranded telomeric region based on nucleotide complementarity. In addition, the budding yeast genome does not contain a gene encoding a TRF-like telomeric protein. The only known ortholog of a human telomeric protein – scRap1p, containing its own Myb domain – seems to fulfill functions divided between TRF proteins and hRap1p in mammalian cells (Marcand et al. 1997, McEachern et al. 2000).

Based on a presence of two repetitive sequences (X and Y'), the chromosome ends in *S. cerevisiae* are generally of one of two types (Fig. 3): those that have only X elements and those that have X and Y' elements. The X element can now be defined as a core X component along with associated variable sequences. The core X is found in some form at all 32 ends. All ends contain at least the X-ACS region and 31 of the 32 ends contain the conserved Abf1p binding site. Most of the core X elements are full length (approximately 475 bp). Between the X elements and the terminal telomere repeats (or between the X and Y' elements) are several small subtelomeric repeat sequences (STRs) that can vary in presence/absence as well as copy number. These contain potential binding sites for Tbf1p. The Y' elements can vary in copy number from 1-4 tandem repeats when present at an end and usually have telomeric repetitive sequences at the centromere proximal end (see the web page of Ed Louis at <http://www.le.ac.uk/ge/ejl12/> for more details).

Indirect genetic evidence suggests that telomeres in *S. cerevisiae* appear to form fold-back structures thus emphasizing the role of telomere looping as a common theme in telomere architecture (de Bruin et al. 2000, de Bruin et al. 2001) (Fig. 4). These results support previous observations from Grunstein's laboratory proposing that the folding back of telomeric DNA allows interaction between Sir protein complexes bound to Rap1p, and those associated with the more internal histones (Grunstein 1997). These complexes form core heterochromatin which can spread through interactions between Sir3p and the histones. A similar looping model has been proposed to explain

silencing in *Drosophila*, with interactions between Polycomb group proteins leading to the formation of a core silencing complex (Pirrotta 1997). In an alternative model, Pryde and Louis proposed that silencing at native ends requires the interaction of telomere-associated Rap1p/Sir2p,3p,4p complexes with proteins bound at subtelomeric, ~450 bp repetitive sequence (called X element), leading to the formation of a region of highly repressive chromatin (Pryde and Louis, 1999). To directly visualize the telomeric structure, we developed a system based on a *S. cerevisiae* minichromosome carrying an array of *lac* operator sequences. To isolate the minichromosome in a sufficient quantity and purity we took advantage of a system provided by Robert Simpson from Pennsylvania State University. The system is based on a *lac* repressor cloned in fusion with a chitin-binding domain separated with an intein sequence. The *lac* repressor-fusion protein is purified from the bacteria by chitin-sepharose thus making an affinity matrix for purification of the *lac* operator containing DNA. The bound DNA is eluted from the beads by DTT-induced intein splicing leading to the fractions that can be checked for purity of the preparation by PCR and inspected by EM. The system should be instrumental not only for visualization of the telomeric structure, but in the long run also for assessment of the positions of candidate telomeric proteins within the budding yeast telosome.

LOOPING AS A COMMON THEME IN TELOMERE ARCHITECTURE II: THE FISSION YEAST

In contrast to *S. cerevisiae*, fission yeast contains a TRF homologue (Taz1p) and similarly to mammalian cells, spRap1 binds to the telomeres indirectly via Taz1p. In addition, the role of spRap1p seems to be restricted to telomeres and thus is distinct from that of scRap1p, which is involved in silencing at both the telomere and mating type locus (Park et al. 2002). Therefore, fission yeast is a suitable model for the study of TRF-mediated telomere transactions and evolution of mammalian-like telosome.

Taz1p (telomere-associated in *Schizosaccharomyces pombe*) was originally found in a one-hybrid screen using telomeric DNA as a target (Cooper et al. 1997). It shares homology to the Myb proto-oncogene DNA-binding domain with both TRF1 and TRF2. In addition, similar to TRF1 and TRF2, it contains a centrally located sequence motif of about 200 amino acids, referred to as the TRF homology (TRFH) domain that is

unique to this gene family and overlaps with a dimerization domain mediating strong homotypic interactions (Bianchi et al. 1997, Broccoli et al. 1997). The possibility that Taz1p functions in fission yeast may encompass those of both hTRF1 and hTRF2 is consistent with the observation that the Myb domain and TRFH domain of Taz1p are more homologous to hTRF1, whereas other regions of Taz1 show higher levels of homology with hTRF2 (Li et al. 2000).

Disruption of the *taz1⁺* gene in *S. pombe* is not lethal and *taz1⁻* haploids grow vegetatively at the same rate as the wild-type cells. Deletion of *taz1⁺* causes a dramatic (about 10-fold) increase in telomere length (Cooper et al. 1997). However, despite the changes in telomere length, *taz1⁻* cells did not exhibit a decrease in viability after extensive sub-culturing (Cooper et al., 1997). In contrast to the vegetative growth, sexual reproduction of *taz1⁻* cells is aberrant due to defective meiosis (Cooper et al. 1997, Cooper et al. 1998, Nimmo et al. 1998)). Detailed analysis of the role of Taz1p in meiotic division revealed that it is necessary for telomere aggregation adjacent to the spindle pole body (SPB) during meiotic prophase. In addition to its role in meiosis, Taz1p is required for the repression of telomere-adjacent gene expression (Cooper et al. 1997), for telomere protection under nitrogen starvation inducing an uncommitted G1 state (Ferreira and Cooper 2001), and for cell cycle progression at 20°C, a temperature at which *Dtaz1* mutants exhibit a G2/M checkpoint delay, chromosome missegregation and DNA double-strand breaks (Miller and Cooper 2003). Taz1p thus represents a telomeric cap that prevents non-homologous end-joining machinery from recognizing telomeres as double-strand breaks.

Similar to the *taz1⁻* mutant, the *rap1⁻* mutant of *S. pombe* is nonessential for mitotic growth, but leads to elongation of telomeres and defective meiosis. Cells disrupted for both *taz1⁺* and *rap1⁺* genes exhibit the same phenotype as single disruptants, suggesting that they function in the same pathway(s) (Chikashige and Hiraoka 2001). Thus Rap1p of fission yeast binds to the telomere through interaction with Taz1p, as found in human cells (Li et al., 2000).

In addition to Taz1p and Rap1p, and similar to higher eukaryotes, other proteins, such Pot1p, Est1p, Ku, and Mre11/Nbs1/Rad50 complex are involved in a formation of higher-order telomere structure in the fission yeast. It is in our interest to assess the direct role of these telomeric components in the formation of fission yeast telosome. However, the size of fission yeast telomeric tracts (<500 bp in *S. pombe* versus >5 kb in mammalian cells) and the low abundance of

telomeres (6 telomeres per fission yeast cell versus 92 telomeres per human diploid cell) exclude the possibility of quantitatively isolating *S. pombe* telomeric fragments and thus assaying for the presence of their structure *in vivo* analogously to mammalian cells (Griffith et al. 1999). Therefore we are trying to reconstitute the fission yeast telosome using a model telomere DNA containing a double-stranded array of fission yeast telomeric repeats in which the single-stranded overhang can be altered in sequence and length. Our ultimate goal is to reconstitute the fission yeast telosome *in vitro* from purified components. We have initiated a study of the ability of recombinant Taz1p to bind and to remodel the fission yeast telomere *in vitro* (Fig. 2C). We believe that the results will provide the first step towards understanding telomere remodeling in fission yeast that, in addition to its evolutionary implications, might be relevant for the understanding of the molecular principles of telomere dynamics in higher eukaryotes.

FINAL REMARKS

In the current textbooks of molecular biology, telomeres are described as structures at the ends of linear DNA molecules required for their complete replication. In addition to their role in DNA synthesis, telomeres must protect the chromosome ends from DNA damage responses such as cell cycle arrest and apoptosis. This feature of telomeres is especially important for cancer cells, which continue proliferating in spite of chromosome aberrations. The telomere capping is probably mediated by several factors acting in parallel, including an active (and novel) role for (i) telomerase, (ii) DNA damage proteins involved in non-homologous end-joining pathway and (iii) overall telomere architecture. The latter mechanism of telomere protection is exemplified by telomere looping represented either by invasion of a 3' telomeric overhang into the duplex region of the telomere (t-loops), or by fold-back structures induced by protein-protein interactions at the telomere. We have developed experimental systems to study both mechanisms in fission and budding yeasts, respectively, with the ultimate goal of answering the question in the title of our presentation.

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