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Protecting the terminus: t-loops and telomere end-binding proteins

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Abstract. Telomeric DNA is composed of a region of duplex telomeric tract followed by a single-strand overhang on the 3′ G-rich strand. The DNA is packaged by proteins that associate directly with the single- and double-strand regions of the telomeric tract and by their associated proteins. This review discusses the evidence that G-strand overhangs are present on both ends of eukaryotic chromosomes and the steps needed to generate these overhangs. The overhangs are protected by specialized G-overhang-binding protein and/or invasion by the overhang of the duplex region of the telomeric tract to form a structure called a 't-loop'. The G-overhang-binding proteins identified from different species are described, and their properties compared. The data supporting the existence of t-loops at native telomeres is discussed, and the conditions required to promote their in vitro formation are presented.

Key words. Telomere; replication; DNA structure; telomere protein; t-loop; TEBP; Pot1; Cdc13.

Introduction

All organisms with linear chromosomes need to protect the DNA terminus from end-to-end joining and degradation. They must also overcome the end-replication problem that arises from the inability of DNA polymerase to replicate the extreme 5′ end of a linear DNA molecule [1]. Eukaryotic cells have evolved a general solution to this set of end-associated difficulties by developing a protective telomeric structure in which repeated sequence DNA is packaged by a group of specialized telomere proteins [2, 3]. In many organisms the repeated sequence DNA is replenished after DNA replication by telomerase, a specialized reverse transcriptase that uses its integral RNA subunit as a template. Although telomere function is conserved across species, upon initial inspection, there appear to be significant differences in the structure of the telomeric nucleoprotein complex. The length of the telomeric tract ranges from <30 nt in some ciliates to ~50 kb in mice, and the overall number and identity of the telomere-specific proteins are quite variable [4]. Despite these apparently major differences in telomere organization, it has become apparent that the essential components of a telomere are quite conserved. In most organisms the telomeric DNA consists of a tract of duplex repeats followed by a much shorter single-strand 3′ overhang. The strand that extends toward the 3′ end of the chromosome is usually G rich, so the overhang is termed the 3′ or G-strand overhang. The duplex DNA is coated by telomere-specific double-strand DNA (dsDNA)-binding proteins, whereas the G-strand overhang is coated by one or more single-strand DNA (ssDNA)-binding proteins. As discussed below, these G-overhang-binding proteins not only are required to protect the DNA terminus from degradation and unwanted DNA repair activities but are also important for recruiting the enzymes needed for telomere replication. Species differ in the role of the double-strand telomere-binding proteins [see Ishikawa review, this issue]; in some they seem to be essential for protecting the DNA terminus [5, 6], whereas in others they are needed for telomere length regulation but seem to be less important for end protection [7, 8].

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Terminal DNA structure

The structure of the DNA at the very end of the telomeric tract is extremely important because it determines how the end of the chromosome is packaged [9]. This packaging by telomere proteins is what allows regulated access to telomerase but protection from other DNA-modifying activities such as nucleases or the DNA repair machinery. Replication of the telomeric tract by the conventional replication machinery is predicted to result in an overhang at one telomere and a blunt end at the other (fig. 1) [10]. The telomere replicated by lagging strand synthesis will end up with a short 3′ overhang after removal of the RNA primer from the terminal Okazaki fragment, and longer overhangs may be generated if this last Okazaki fragment is initiated internal to the DNA terminus. In contrast, the telomere replicated by leading-strand synthesis will have a blunt end if the parental DNA is copied all the way to the 3′ terminus or a 5′ overhang if synthesis terminates prematurely [11]. If this asymmetric structure were maintained throughout the cell cycle, the two opposite ends of a chromosome would end up being packaged quite differently. A second outcome is that only one telomere would be a substrate for telomerase because telomerase is incapable of extending a blunt-end duplex or a 3′ underhang [12]. Determining whether the predicted asymmetry exists in nature has proved difficult because of the low abundance of telomeres and the repeated nature of the telomeric sequence. A combination of electron microscopy (EM) and oligonucleotide hybridization and/or primer extension experiments has led to a consensus, however, that most eukaryotic chromosomes have 3′ overhangs on both ends but that species differ in overhang length. The most

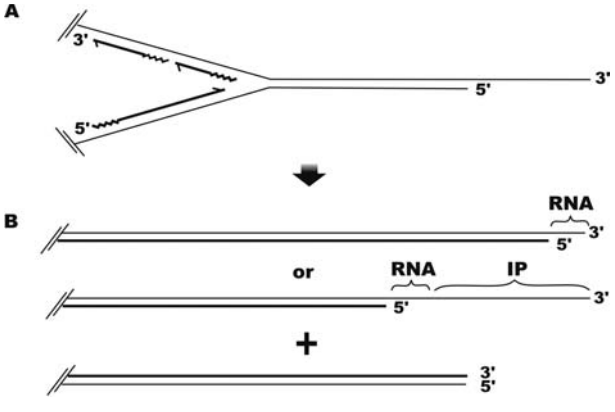

Figure 1. Replication of the telomeric tract by leading- and lagging-strand synthesis generates two different DNA termini. (*A*) Replication fork moving toward the DNA terminus. (*B*) Final products. Leading-strand synthesis generates a blunt end (lower product), whereas removal of the RNA primer (represented by the zigzag line) from the lagging strand generates a short 3′ overhang (upper product, region labeled RNA). Longer overhangs may be generated if the final Okazaki fragment is laid down internal to the DNA terminus (middle product, region labeled IP).

direct evidence that overhangs are present on both ends of a chromosome has been obtained from protists that have either small chromosomes or short telomeres. Entire minichromosomes can be isolated from trypanosomes and examined by EM, allowing regions of ssDNA to be visualized by decoration of the chromosomes with *Escherichia coli* SSB protein [15]. Such an analysis revealed the presence of bound SSB tetramers and hence overhangs at both ends of full-length minichromosomes. On the basis of the number of tetramers bound, trypanosome overhang length was estimated to be in the 75–225-nt range, but shorter overhangs may also exist, as SSB requires a minimum of 75 nt to bind. Characterization of overhang length is most advanced for ciliated protozoa, because telomeres from *Oxytricha* and *Euplotes* are short enough to be sequenced directly [16], whereas a recently developed ligation-mediated primer-extension technique has allowed analysis of *Tetrahymena* G-strand overhangs at nucleotide resolution [9]. *Oxytricha* and *Euplotes* have overhangs on both telomeres that are exactly 14 and 16 nt, respectively. In *Tetrahymena* the overhangs differ in length by multiples of 6 nt, but the majority are 14–15 or 20–21 nt long. They are present on both telomeres and show little length variation during the cell cycle.

Budding yeast cells also have short overhangs on both telomeres, but overhang length changes during the cell cycle [13]. During late G1 overhangs are only ~15–20 nt in length [R. Wellinger, personal communication], but during late S phase, the time of telomere replication, they become ≥30 nt [13]. In contrast, mammalian telomeres have G-strand overhangs in the 150–350-nt range that are present throughout the cell cycle [14, 17–19]. The actual length depends on cell type and is correlated with the length of the Okazaki fragments [14, 17]. Several studies indicate that the G-stand overhangs are present on both telomeres, although their lengths may be quite different [17, 20]. The telomeres of plant chromosomes may also differ in overhang length; the longest are in the 20–30-nt range [21] [D. Shippen, personal communication]. Interestingly, overhangs have recently been detected on the telomeric C strands of human cells [11]. These are present transiently during S phase and may result from stalling of the replication fork on the leading-strand telomere.

For a chromosome to have a G-strand overhang on both telomeres requires that the leading-strand telomere be subject to some form of DNA processing, for example resection of the C strand by a nuclease or extension of the G strand by telomerase after a helicase or nuclease has generated a few unpaired nucleotides. The first evidence that nucleases participate in overhang generation was obtained by examination of chromosomes from telomerase-deficient yeast. These were found to have G-strand overhangs on both telomeres, indicating that the C strand of the leading-strand telomere must have been resected by a nuclease [22, 23]. More recent work with *Tetrahymena*

has demonstrated that at least two specific processing events are required to generate overhangs of the correct length and sequence. One event involves resection of the C strand to a specific position within the C_4A_2 repeat, and the other involves cleavage of the G strand so that at least two nucleotides are removed from the 3′ terminus [9] [N. K. Jacob and C. Price, unpublished results].

Because generating overhangs on both ends of a chromosome requires significant effort, having this type of terminal structure is probably advantageous. The advantage may be that telomeres with G-strand overhangs are poor substrates for nonhomologous end joining and hence are protected from end-to-end fusions [24], but telomeric DNA is vulnerable to unregulated nuclease digestion [25], and single-strand overhangs are particularly easy to remove [6]. This strategy for preventing end-to-end fusions will therefore only be successful if the overhangs are somehow protected. As outlined below, cells seem to have evolved two approaches for protecting telomeric overhangs: binding by single-strand telomere-binding proteins and the formation of t-loops. Although most G overhangs have the capacity to form G quartets and other G-G base-paired structures in vitro [26], there is currently no evidence that such structures are used to protect the overhangs in vivo.

The TEBP/Pot1 family of G-overhang-binding proteins

Studies of telomere end protection became possible when the first telomere protein was isolated from the ciliate *Oxytricha nova* [27–29]. Ciliates such as *Oxytricha* and *Euplotes* have literally millions of telomeres, so the telomere end-binding proteins (TEBPs) are very abundant. This abundance allowed the *Oxytricha*, and later the *Euplotes,* TEBP to be purified as DNA-protein complexes by means of a single gel-filtration step. Subsequent analysis revealed that both the *Oxytricha* and the *Euplotes* proteins bind single-stranded G_4T_4 repeats with great specificity, and once they are bound, the DNA is heavily protected from nuclease digestion (see table 1) [28, 30]. They also have a strong preference for the DNA terminus, recognizing the 5′-G_4 or 5′-G_2 sequence found at the 3′ end of the G-strand overhang. The *Oxytricha* TEBP (OnTEBP) is a heterodimer composed of a 56-kDa α subunit and a 41-kDa β subunit. Studies with recombinant protein revealed that the α subunit binds DNA on its own, whereas the β subunit does not. Although the α and β subunits of the *Oxytricha* heterodimer are tightly associated, the *Euplotes* protein has only been isolated as a monomer [31]. This 51-kDa protein shares extensive sequence identity with the *Oxytricha* α subunit over an ~300-amino-acid region that comprises the DNA-binding domain [32].

The *Oxytricha* TEBP has been crystallized both as an α-β-DNA ternary complex [33] (shown in fig. 2) and as an α homodimer complexed to DNA (the $[\alpha\text{-ssDNA}]_2$ complex [34]. The structure of the ternary complex explains why the TEBP is so effective at protecting the DNA terminus, because the terminal 12 nt lie in a deep cleft between the α and β subunits [33]. The individual bases are accommodated by a series of pockets within the cleft, and the DNA backbone traces an irregular path; the 3′ end

Table 1. Comparison of telomeric G-strand-binding proteins.

Gene family	Organism	Name of proteins	Accession numbers	Region of sequence similarity	Sequence identity (similarity)	DNA binding G strand	Specificity DNA terminus	Telomere function	Reference
TEBP	*O. nova*	OnTEBP	P29549	28–150		yes	yes	yes	24, 31, 34, 39, 71, 72
	E. crassus	EcTEBP	Q06184	4–121	46 (61)	yes	yes	yes	70, 83
Pot1	*S. pombe*	SpPot1	NP_594453	21–137	23 (35)	yes	some	yes	2, 3
	H. sapiens	HsPot1	Ak001935	1–109	23 (35)	yes	ND	yes	2, 3
	M. musculus	MmPot1	AAH16121	1–109	24 (39)	ND	ND	ND	3
	G. gallus	GgPot1	n/a	145–253	25 (36)	yes	ND	yes	Wei and Price, unpublished
	A. thaliana	AtPot1	NP_196249	2–111	29 (45)	ND	ND	ND	3
	A. thaliana	AtPot1	NP_17859	1–117	26 (43)	ND	ND	ND	3
Cdc13	*S. cerevisiae*	Cdc13	P32797		NC	yes	no	yes	1, 41, 48, 59, 64
Est1	*S. cerevisiae*	Est1	P17214		NC	yes	yes	(yes)	21, 22, 82

A. thaliana has two Pot1-like genes. NC, not conserved; ND, not determined. Sequence identity and similarity with DNA binding domain of OnTEBP, obtained with Seqweb (GAP, scoring matrix: Blosum 35). Telomere function indicates evidence for telomere function or localization from DMS footprinting (OnTEBP, EcTEBP), genetic analysis (SpPot1, Cdc13, Est1), immunolocalization (HsPot1, GgPot1), or telomerase subunit (Est1).

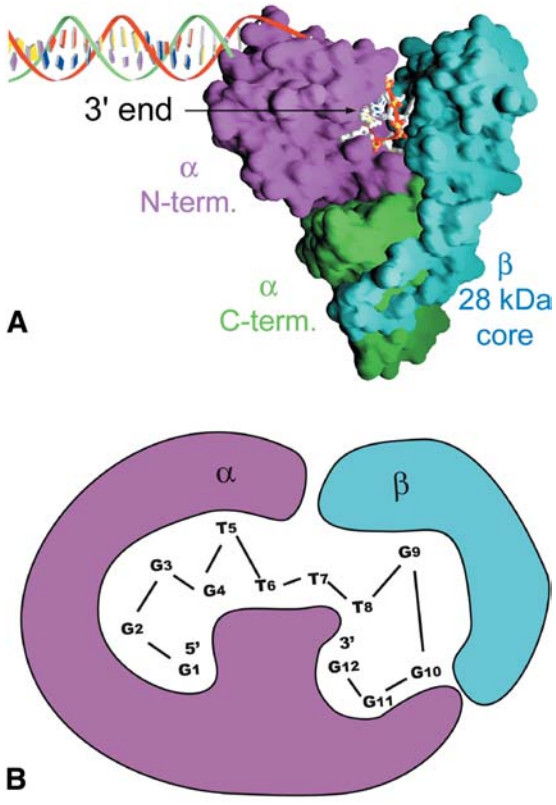

Figure 2. Structure of the OnTEBP α-β-DNA ternary complex. (*A*) Crystal structure. The telomeric DNA sits in the deep groove formed by the β subunit (blue) and α subunit amino terminus (pink) (courtesy of M. Horvath [33]). (*B*) Schematic showing interactions between the telomeric DNA and the α and β subunits.

forms a loop that leaves the terminal nucleotide buried deep within the heterodimer. The complex is stabilized by an unusually extensive array of stacking interactions between the bases and aromatic amino acids. The complex is also unusual in that it contains four OB (oligonucleotide/oligosaccharide-binding) folds, three forming the cleft that binds the telomeric DNA and a fourth forming the interaction surface between the α and β subunits. Interestingly, the structure of the α homodimer has revealed a second and very different mode of DNA binding [34]. In the homodimer, the second α subunit covers part of the surface used to bind DNA in the α-β-DNA ternary complex. As a result, the DNA terminus is left exposed, and multiple homodimers can bind along a single DNA molecule. Because the surface that interacts with the β subunit in the α-β-DNA ternary complex remains accessible in the $(\alpha\text{-DNA})_2$ dimer, the TEBP may first bind G overhangs as a homodimer but then associate with β and be converted into the more protective α-β heterodimer. It has not been possible to explore the in vivo role of the TEBP in chromosome end protection because *Oxytricha* is not amenable to genetic studies, but dimethylsulfate (DMS) footprinting indicates that the α-β-DNA ternary complex is almost certainly present at native *Oxytricha* telomeres [29, 35].

Isolation of the ciliate TEBPs raised the expectation that homologous proteins would be present in other eukaryotes. A protein with similar DNA-binding properties was subsequently detected in *Xenopus* extracts [36], but repeated attempts to clone TEBP homologs from yeast and mammalian cells were unsuccessful. Moreover, Cdc13p, a protein that shares little sequence identity with the ciliate TEBPs, was identified as the *Saccaromyces cerevisiae* telomeric G-strand-binding protein [37, 38]. TEBPs therefore initially appeared not to be evolutionarily conserved. Homologs of the OnTEBP β subunit have still only been identified in closely related ciliates, but completion of the *S. pombe* and human genome projects eventually revealed a family of proteins that have homology to the OnTEBP α subunit. These proteins were named Pot1 for protection of telomeres [39]. At the same time, nuclear magnetic resonance (NMR) analysis of the Cdc13p DNA-binding domain revealed considerable structural homology to the OnTEBP α subunit, suggesting that these two proteins may be more conserved than originally thought (see below).

Database searches have since shown that Pot1-like proteins are present in a wide range of organisms, including mammals, plants, yeasts and microsporidia [40]. Although the overall level of sequence conservation between family members is quite low, they all have an ~120-amino-acid region near the N terminus that shares sequence identity with a portion of the DNA-binding domain of the ciliate TEBP α subunit (table 1). The *S. pombe* Pot1 has ~23% identity and 35% similarity to this region of the *Oxytricha* TEBP, whereas the same regions of human and *S. pombe* Pot1 proteins are ~28% identical and 44% similar [39] (table 1). The DNA-binding specificity of the *Schizosaccharomyces pombe* and human Pot1 proteins resembles that of the *Oxytricha* and *Euplotes* TEBP [28, 39]. Both Pot1 homologs bind to ssDNA corresponding to the G-rich strand of their respective telomeric sequence but do not bind dsDNA or ssDNA corresponding to the telomeric C strand. Moreover, SpPot1 exhibits a preference for the 3′ end of a telomeric oligonucleotide. Interestingly, the *hPot1* gene is alternatively spliced to give at least five different splice variants [40]. The largest splice variant encodes a 71-kDa protein, whereas the smallest encodes a 5-kDa protein. When the variants encoding the DNA-binding domain are in vitro translated, the proteins exhibit surprisingly different affinities for G-strand telomeric DNA. The hPot1 variants may therefore have distinct in vivo functions.

Evidence that the Pot1 family members are *bona fide* telomere proteins has been obtained from immunolocalization studies in human cells that demonstrated a telomeric distribution and in vivo studies in *S. pombe* that demonstrated an essential role in telomere maintenance

[39, 40]. The telomeric distribution was demonstrated by transient transfection of human cells with a tagged version of the hPot1 gene. The result was a punctate nuclear distribution in interphase cells in which many of the spots colocalized with the known telomere proteins hRap1 and hTRF2. Deletion of the SpPot1 resulted in a cell-division defect leading to elongated cells that failed to divide further and a high incidence of chromosome missegregation. Further analysis revealed that the deletion mutants had undergone rapid loss of telomeric and subtelomeric sequences and chromosome circularization. Thus, as might be expected of a telomere end-binding protein, SpPot1 is essential for chromosome stability.

Cdc13 and telomere maintenance

The *S. cerevisiae* protein Cdc13 was first shown to play a role in telomere protection when a temperature-sensitive mutant was found to cause Rad9-dependant cell-cycle arrest [41]. Analysis of cells grown at the semipermissive temperature revealed an accumulation of ssDNA and preferential degradation of the telomeric C strand [42, 43]. *CDC13* was subsequently identified in a screen for mutations that let to progressive telomere shortening (the ever shorter telomere or Est$^-$ phenotype), suggesting that it also played a role in telomerase-mediated telomere maintenance [38, 44]. Cdc13 has since been shown to have multiple separate functions in telomere maintenance; these include an essential capping or end-protection function, telomerase recruitment, telomerase repression, and coordination of G- and C-strand synthesis. It achieves these functions by binding the G-strand overhang and acting as a landing pad that recruits a series of unique protein complexes each of which performs a different task [45].

Cdc13 is an essential 105-kDa multidomain protein that interacts with ssDNA, the telomerase subunit Est1, DNA polα-primase and the telomere proteins Stn1 (*s*uppressor of cdc *t*hirtee*n*) and Ten1 (*te*lomeric pathways in association with St*n*1 number *1*) (fig. 3A). Although the regions involved in the Stn1 and polα interactions have only been partially mapped [46, 47], the Est1 binding domain has been localized to a ~15-kDa region that is sufficient to recruit the telomerase complex to the telomere [48]. The DNA-binding domain (DBD) consists of a ~24-kDa region that binds telomeric DNA with essentially the same specificity as the full-length protein [49–51].

Although Cdc13 resembles the TEBP/Pot1 proteins in that it binds specifically to G-strand telomeric DNA [37, 38], it does not have a preference for the DNA terminus. It has a minimum binding site of 11 nt, and in vitro multiple Cdc13 molecules can load on an oligonucleotide with one molecule binding per 11 nt [50]. One-hybrid and ChIP (chromatin immunoprecipitation) experiments have shown that Cdc13 is present at telomeres throughout the cell cycle [52, 53], but the amount of protein that is present increases greatly in late S phase. This pattern is consistent with polymerization of Cdc13 along the extended G overhangs that are present at that time. Although Cdc13 recruits telomerase to the telomere, the lack of specificity for the DNA terminus means it cannot be responsible for positioning the enzyme complex at the end of the overhang. This function is probably performed by the telomerase subunit Est1 in a step that is proposed to occur after recruitment of telomerase to the chromosome end [54, 55]. Est1 binds G-strand telomeric DNA with lower affinity than does Cdc13, but it does have a preference for the terminus [56].

Structural analysis of the Cdc13 DNA binding domain ($Cdc13_{DBD}$) by NMR spectroscopy revealed that the DNA binding motif is composed of a single OB fold [49]. Although the Cdc13 OB fold and the three OB folds found in the OnTEBP DNA binding domain show no apparent sequence conservation, they are structurally very similar (see fig. 3B). This finding suggests that the OB fold is a generally conserved structural element that is used by telomere proteins to bind G-rich ssDNA. OB folds consist of a β barrel formed by two orthogonally packed three-stranded antiparellel β sheets. The ligand interactions typically occur through the loops that connect the β strands [57]. As shown in figure 3B, the $Cdc13_{DBD}$ can be superimposed over the most N terminal fold of the OnTEBP α subunit with a 2.2 Δ standard deviation, but when the amino acids involved in DNA binding are mapped on the TEBP and Cdc13 structures, significant differences can be seen in the interaction surfaces on the loops. Thus, the OB fold seems to resemble the RNA-binding RRM (RNA recognition motif) where a conserved $\alpha\beta$-core structure provides an optimal surface for RNA binding, but much of the sequence and structure specificity is provided by the loop regions and adjacent structural elements [58, 59].

The separate roles of Cdc13 in telomere end protection and telomerase recruitment or repression have been demonstrated by an elegant series of experiments using Cdc13 fusion proteins and various *cdc13* and *est1* mutants [46, 48, 54]. Deletion of *CDC13* is normally lethal, whereas some mutations cause telomere lengthening and others cause telomere shortening. The lethality of a cdc13-Δ strain can be counteracted by fusion of the $Cdc13_{DBD}$ to an N-terminal fragment of the telomere protein Stn1 [48]. Although the fusion protein restores both viability and protection of the telomeric C strand, the cells still display progressive telomere shortening (the Est$^-$ phenotype). Thus, the $Cdc13_{DBD}$-Stn1 fusion restores end protection but not telomerase recruitment, demonstrating that these are separable functions. The protective function of Cdc13 appears to be achieved through the delivery of a multiprotein complex that contains not only Stn1 but also a novel protein Ten1 [60] (see fig. 3C). Ten1 binds both Cdc13

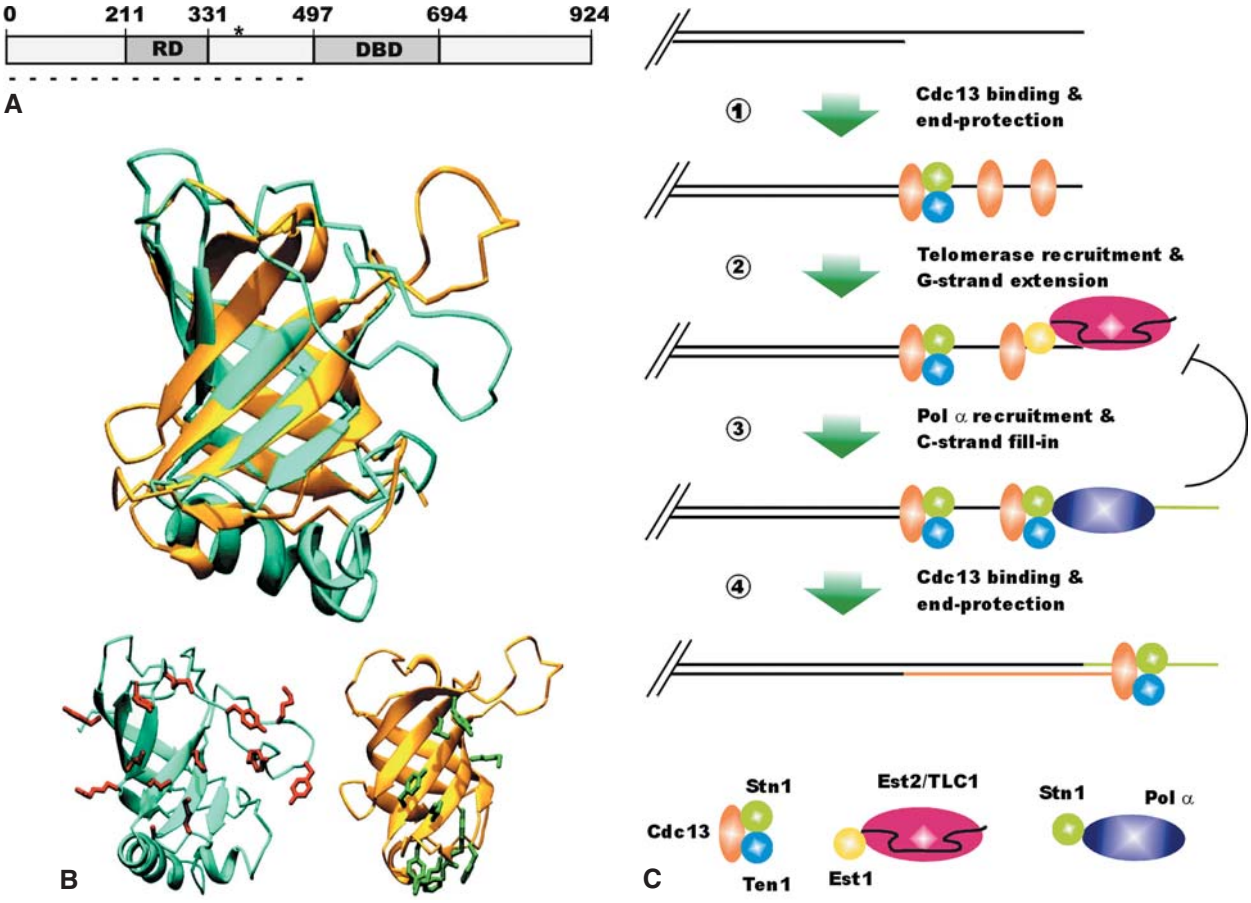

Figure 3. Structure and function of Cdc13. (*A*) Diagram depicting the locations of the various Cdc13 interaction domains. RD, Est1 recruitment domain; DBD, DNA-binding domain; the interrupted line marks regions to which Pol α interactions have been mapped; the star marks the site of the original Cdc13-1 ts mutation; this mutation also disrupts Stn1 interaction. (*B*) Structural comparison of the OB folds from the $Cdc13_{DBD}$ and the N terminus of the OnTEBP α subunit. Upper portion shows the overlay of the two folds, $Cdc13_{DBD}$ is in cyan, OnTEBP is in gold. Lower portion shows the DNA-binding interfaces of the two OB folds. Contact residues of the $Cdc13_{DBD}$ are shown in red (left), and those of the OnTEBP are shown in green (right) (courtesy of D. Wuttke [49]). (*C*) Model for how Cdc13 complexes modulate telomere end protection, telomerase recruitment, telomerase repression, and coordination of G- and C-strand synthesis. Step1, Cdc13 binds to the extended G-strand overhangs that are created in late S phase, delivering the protective Cdc13/Stn1/Ten1 complex to the junction with the duplex DNA. Step 2, Cdc13 recruits telomerase by means of an interaction with Est1, and new telomeric repeats are synthesized. Step 3, the Est1 interaction is replaced by an Stn1 interaction, so telomerase is displaced and further extension of the G strand is prevented. DNA pol α-primase is recruited, and resynthesis of the telomeric C strand begins. Step 4, C-strand synthesis is completed, and a protective Cdc13/Stn1/Ten1 complex is positioned on the remaining short 3′ overhang (adapted from [46]).

and Stn1, and *ten1*$^-$ mutants exhibit the same loss of telomere length regulation and accumulation of long G-strand overhangs as some *cdc13*$^-$ and *stn1*$^-$ mutants.

The role of Cdc13 in telomerase recruitment was proven by demonstration that Cdc13-Est1, $Cdc13_{DBD}$-Est1 and Cdc13-Est2 fusions (Est2 is the telomerase catalytic subunit) rescued the Est$^-$ phenotype of both *cdc13* and *est1* mutants [54]. More recent experiments have shown that normal telomere function can be restored to *cdc13* null mutants by expression of both the $Cdc13_{DBD}$-Stn1 fusion described above and a minimal Cdc13 that consists of a fusion between the DBD and the Est1 recruitment domain ($Cdc13_{DBD-RD}$) [48]. The interaction between Cdc13 and Est1 is therefore necessary and sufficient to restore telomerase recruitment and prevent the Est$^-$ phenotype.

Although Cdc13 recruits telomerase to the telomere through the Est1 interaction, it also limits how much telomerase extends the G-strand overhang [46, 61, 62]. This repressive function seems to be mediated by Stn1, because both Cdc13-Stn1 fusions and Stn1 overexpression prevent telomere growth in *cdc13* mutants that have unusually long telomeres. Stn1 overexpression also prevents the accumulation of the abnormally long G overhangs that are observed in certain Cdc13 and DNA polα mutants [46]. These long G overhangs have been proposed to result from a loss of coordination between the leading- and lagging-strand replication machinery, suggesting that the Cdc13-Stn1 interaction must also be important for coordinating G- and C-strand synthesis.

The contradictory role of Cdc13 in both telomerase recruitment and repression can best be explained by a temporally regulated switch in Cdc13 interactions (fig. 3C) [46]. Initially, telomerase is recruited to the telomere by the Cdc13-Est1 interaction, which is then replaced by the Cdc13-Stn1 interaction. The switch to Stn1 binding would displace telomerase and allow fill-in synthesis of the C-strand by DNA pol α. Although this model provides an elegant explanation for much of the available data, the in vivo situation is probably even more complicated. The DNA-repair protein Ku has also been implicated in telomerase recruitment [61, 63], but ChIP experiments indicate that Est2 (the telomerase catalytic subunit) is actually present at the telomere throughout the cell cycle [53]. This result raises the possibility that telomerase recruitment is a multistage process. It is also unclear how the Cdc13 complexes achieve end protection as opposed to coordination of leading- and lagging-strand synthesis, and further studies of this process are likely to reveal interesting twists and complexities.

hnRNPs: a role in telomere biology?

The heterogeneous nuclear ribonuclear proteins (hnRNPs) are a large family of nucleic acid-binding proteins that bind to premessenger RNAs (pre-mRNAs) in the nucleus and mediate many different aspects of mRNA maturation and turnover [64]. Intriguingly, a subgroup of hnRNPs has been found to bind telomeric DNA and/or telomerase, raising the possibility that they also have a role in telomere metabolism. The first evidence for hnRNP association with telomeric DNA was obtained during attempts to isolate mammalian homologs of the OnTEBP from nuclear extracts [65, 66]. hnRNP A1, A2-B, D and E were all shown to bind single-strand oligonucleotides containing T_2AG_3 repeats, but although these proteins bind DNA in a sequence-specific manner, they have a higher affinity for RNA, and some bind specifically to 3′ splice sites. Subsequent studies with purified proteins showed that hnRNP A1 and UP1 (a fragment of A1) protect G-strand DNA from degradation, whereas multiple different isoforms of hnRNP D not only bind to single-stranded G-strand oligonucleotides but also destabilize G-G pairing [67, 68]. The latter finding led to the suggestion that hnRNP D might remove structures formed by folding of G overhangs and facilitate their extension by telomerase.

Immunoprecipitation and pull-down assays indicate that a number of hnRNPs also associate with telomerase. hnRNP A1, UP1, D, C1, C2 and La all copurify with telomerase activity [67, 69–72], and the interaction with C1, C2 and La has been mapped to specific binding sites on the telomerase RNA [69, 70]. In vivo studies point to a role for the hnRNPs in both stimulation and repression of telomerase. Mouse cells deficient in A1 have unusually short telomeres, but restoring A1 expression results in telomeres of normal length [72]. Thus, A1 somehow promotes telomerase activity in vivo even though A1 binding to telomeric oligonucleotides prevents their extension by telomerase in vitro [68]. In contrast, overexpression of La causes telomere shortening, indicating that La somehow antagonizes telomerase activity [70].

Although the experimental data clearly show that a subset of the hnRNPs can bind to telomeric G-strand DNA in vitro, the question remains as to whether they actually do so in vivo. On one hand, they are abundant nuclear proteins that are likely to have access to telomeric DNA. Moreover, some can bind to both telomerase and the G-strand overhang, suggesting that they could play a role analogous to that of Est1 in recruiting telomerase to the telomere [71]. On the other hand, many cells contain Pot1, a *bona fide* telomere protein that binds the same substrate as the hnRNPs. Therefore, the hnRNPs may not be needed at telomeres, and hnRNP-binding could be prevented by Pot1 competition. Moreover, many of the hnRNPs bind both DNA and RNA by means of a conserved RRM RNA-binding motif [67, 73]. Because these proteins have a greater affinity for RNA than for DNA, they could be tailored to bind specific sequences in pre-mRNA (e.g. splice site junctions), and their ability to bind telomeric DNA in vitro may merely reflect a lack of specificity for DNA over RNA. Finally, although telomere length is clearly altered by changes in the level of hnRNP A1 and La expression, this effect could be indirect, because A1 and La influence many aspects of mRNA metabolism and hence may affect the expression of many different genes.

t-loops and other higher-order structures

Although telomeres can encompass many kilobases of DNA, they appear to form quite compact nucleoprotein complexes as a result of looping or folding of the telomeric tract. Evidence for telomere folding was first obtained from *S. cerevisiae* during ChIP experiments with the telomere protein Rap1 [74]. Although RAP1 binds specifically to telomeric DNA, it was found to coimmunoprecipitate with subtelomeric sequences, suggesting that telomere-bound Rap1 is brought close to the subtelomeric DNA by folding or looping of the telomeric tract. Additional evidence for folding was obtained when gene expression was detected from a promoterless marker gene located in the subtelomeric region that had a strong promoter placed downstream and adjacent to the telomere [75]. The gene expression indicated that the telomere may somehow be folded so that the telomere-proximal promoter became positioned upstream of the marker gene.

Although the folding of *S. cerevisiae* telomeres appears to be stabilized by interactions between proteins that bind the telomeric and subtelomeric sequences, looping or folding of telomeres can also be mediated by nucleic acid interactions. Electron microscopy of deproteinized telomeric DNA isolated from organisms with longer telomeres has revealed the presence of large duplex DNA loops at one end [76]. These telomere or t-loops result from the G-strand overhangs becoming tucked into a more centromere-proximal region of the telomeric tract (fig. 4). They seem to be conserved structures, as they have been observed on chromosomes from a wide range of organisms spanning the plant and animal kingdoms; these include humans, mice, ciliates, trypanosomes and peas [15, 76, 77] [J. Griffith, personal communication]. The loops range from 500 bp to 18 kb in length, and in mammalian cells loop size is well correlated with telomere length; the loops are generally a few kilobases shorter than the telomeric tract [76]. In trypanosomes, however, most t-loops are less than 1.5 kb, even though the telomeres are 10–20 kb in length [15]. Regulation of the loop size therefore appears to be species specific.

t-loops were first discovered during inspection of structures formed after incubation of the human telomere protein TRF2 with artificial telomere substrates generated from cloned telomeric DNA [76]. When the telomere substrate had a 3′ G-strand overhang, a striking number of lariat- or lasso-type molecules were observed that had recombinant TRF2 bound at the junction between the loop and the tail. Although formation of these t-loops depended on TRF2, they were not simply held together by TRF2-DNA interactions, because once formed they could be visualized on deproteinized DNA that had been cross-linked with psoralin. In a study examining whether t-loops might also be present at natural telomeres, EM was performed on human and mouse DNA that had been enriched for telomeric restriction fragments. Similar loop structures were seen on 15–40% of the DNA molecules provided they had been psoralin cross-linked prior to DNA isolation. When the cross-linking step was omitted, loops were observed on only 2–5% of the DNA molecules, indicating that the structure is quite labile. Evidence that the loops are present on telomeric restriction fragments rather than contaminating nontelomeric DNA was obtained by decoration of the telomere-enriched DNA with the dsT_2AG_3-binding protein TRF1. In one DNA preparation, in which 40% of the molecules had t-loops, 80% of the molecules were decorated with TRF1, so at least 63% (and perhaps all) of the t-loops contained telomeric DNA [76].

Several lines of evidence indicate that t-loops are formed when the G-strand overhang invades the duplex T_2AG_3 repeats to make a displacement or d-loop. First, interstrand cross-linking by psoralin requires the precise positioning of T residues on opposing strands, arguing that the G-strand overhang is base paired with an upstream region of the telomeric tract. Second, when t-loop-containing preparations of mammalian DNA were decorated with *E. coli* SSB, regions of ssDNA were identified at the junctions between the loops and tails. Third, studies of t-loop formation using recombinant TRF2 and artificial telomere substrates indicated that loops will not form if the substrates are blunt ended, or have 5′ overhangs or 3′ overhangs that lack T_2AG_3 repeats [78]. Therefore, a true G-strand overhang is absolutely required. Surprisingly, only one T_2AG_3 repeat is needed for t-loop formation providing this single repeat is positioned directly adjacent to the duplex telomeric DNA. Longer overhangs that have nontelomeric sequences 3′ to the single T_2AG_3 repeat also

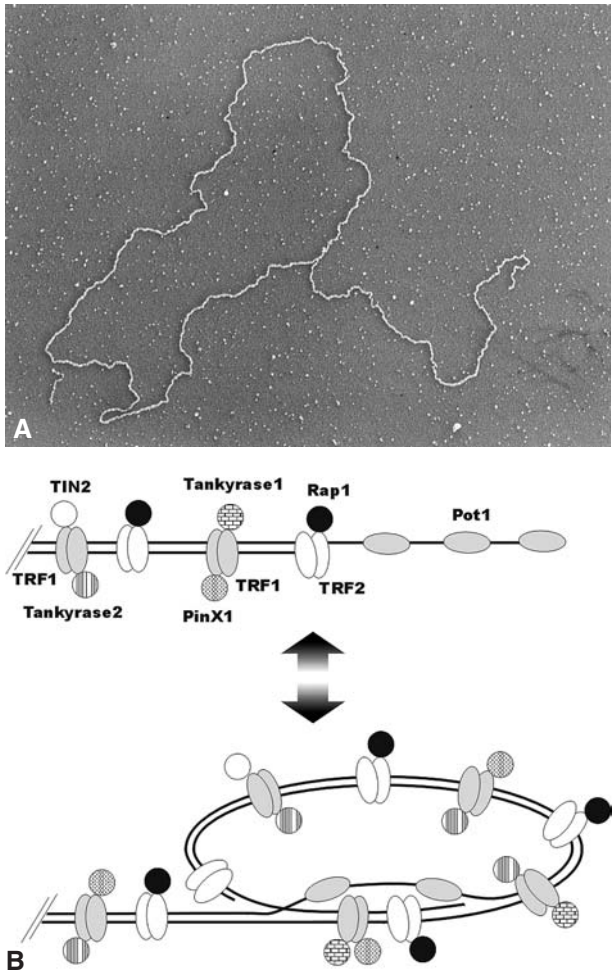

Figure 4. t-loops. (*A*) Electron-microscope image of an 18-kb t-loop from HeLa cells (courtesy of J. Griffith). (*B*) Cycling of telomeres between open and closed states. The upper image depicts a telomere that is extended and in the open state. The lower image depicts a t-loop and a telomere in the closed state. The G-strand overhang is shown invading the duplex telomeric tract to form a d-loop. The various telomere-binding and telomere-associated proteins are shown bound to both the open and closed telomeres. For clarity, only a few protein molecules are shown; the DNA in a native telomere may be completely covered with protein.

form loops with almost the same efficiency as molecules with overhangs that contain only T_2AG_3 repeats. Although a 6-nt T_2AG_3 overhang is sufficient to allow t-loop formation, the DNA duplex is probably invaded by a longer stretch of G-strand DNA, because a 6-nt d-loop should not be stabilized by psoralin cross-linking when the cross-link frequency is only ~1 per 100 bp. DNA adjacent to the overhang may therefore become unpaired during the strand-invasion step.

Exactly how TRF2 mediates t-loop formation is unclear, because this protein binds to duplex telomeric DNA, but it does not bind ssDNA, and it lacks recognizable helicase motifs [78, 79]. However, EM studies have shown that recombinant TRF2 binds preferentially at the terminus of a DNA duplex that has a region of ssT_2AG_3 sequence adjacent to the duplex telomeric tract [78]. It therefore resembles a helicase in that a single-strand tail of defined polarity is preferred for loading. Moreover, the TRF2 observed at t-loop junctions exists not as a simple dimer but as an oligomer of on average ~10 dimers. These observations have led to the suggestion that TRF2 functions rather like RecA, in that the TRF2 oligomer promotes a homology search and then insertion of the G overhang into the telomeric duplex.

Whether mediated by protein or nucleic-acid interactions, looping or folding of the telomeric tract to form a compact nucleoprotein complex is clearly an excellent way to hide the DNA terminus from the DNA repair machinery. It could also provide a means to regulate telomerase access through folding and unfolding of the complex. Such regulation is required because telomerase is present in many cell types throughout the cell cycle [80, 81]. The obvious utility of telomere folding and unfolding has led to the idea that telomeres cycle between open and closed states (fig. 4B); the unfolded structure is the open state, and the compact folded structure the closed state [82, 83]. Presumably, the closed state would exist during G1, G2 and M of the cell cycle, when access to the DNA terminus is neither required nor desirable. The telomere would cycle into the open state during S phase, when access to the terminus becomes essential for telomere replication. t-loops provide a particularly ingenious mechanism for burying the DNA terminus and creating a closed telomere complex, because formation of the d-loop simultaneously renders the G overhang less susceptible to nuclease digestion and initiates telomere folding. The resulting t-loop is probably made even more compact by protein-protein interactions [76].

The discovery of both t-loops and Pot1 in many different organisms raises an interesting puzzle. If telomeres spend most of the cell cycle packaged into t-loops, why is Pot1 needed? One possibility is that Pot1 is needed only transiently during replication, when the telomere cycles into the open conformation, but immunolocalization experiments indicate that substantial amounts of Pot1 are present at telomeres throughout G1, S and G2 of the cell cycle [C. Wei and C. Price, unpublished]. Pot1 may therefore also bind to the displaced G strand that results from t-loop formation. Cycling from the closed to open conformation is essential if telomerase and the G-overhang processing nucleases are to gain access to the DNA terminus. This opening up of the closed conformation may be achieved by a combination of cell-cycle-triggered displacement of double-strand telomere-binding proteins and passage of the replication fork through the t-loop structure [84]. The need to undo t-loops during S phase could explain the function of one or more human helicases that affect telomere maintenance. Both the Bloom's and Werner's helicases can act on unusual DNA structures, and for unknown reasons, mutations in either protein lead to telomere shortening [85, 86]. Perhaps the additional activity of these helicases is needed for replication-fork progression through a t-loop d-loop [87].

Although t-loops are an intellectually satisfying and effective source of telomere end protection, evidence for their existence is based solely on electron microscopy of cross-linked DNA, so additional experimentation is needed to demonstrate that they are truly biologically relevant structures. Furthermore, they may not exist in all organisms. Although EM studies of *Oxytricha* chromosomes revealed t-loops on the long telomeres of *Oxytricha* micronuclear chromosomes, they were not observed on the short 20–28-bp macronuclear chromosomes [77]. Thus, binding of the G overhang by the OnTEBP $\alpha\beta$ heterodimer provides an alternative mechanism for telomere end protection. Whether or not t-loops are present on the chromosomes of organisms such as *S. cerevisiae* and *Tetrahymena* that have intermediate-length telomeres remains an open question. In theory 200–300 bp of telomeric duplex and a short G-strand overhang should allow t-loop formation, but the telomeric DNA of these organisms is not amenable to psoralin cross-linking (they lack the opposing T residues required for interstrand cross-links), so resolution of this question may have to await development of an alternative technique for t-loop visualization.

Conclusions

Cross-species comparison of telomeric DNA structure and terminus-binding proteins has revealed that G-strand overhangs and G-overhang-binding proteins are a critical aspect of telomere end protection in a wide array of organisms. G overhangs are generally present on both chromosome ends, and specialized proteins have evolved to bind these overhangs. Many organisms have G-overhang-binding proteins that have homology to the DNA binding domain of the *Oxytricha* TEBP, suggesting that this DNA binding motif is uniquely suited for telomere protection.

As in all aspects of telomere biology, however, there are variations on a theme, because the *S. cerevisiae* overhang-binding protein Cdc13 has little apparent sequence homology to the OnTEBP. Moreover, the region of sequence identity between the OnTEBP and the Pot1 family of proteins is limited to a short domain. Nonetheless, the OnTEBP, Pot1 family and Cdc13 appear to be functional homologs. Despite the lack of sequence identity, Cdc13 may also be a structural homolog because, like the OnTEBP, it uses an OB fold for nucleotide recognition and binding.

As with the terminal DNA structure and telomere proteins, packaging of telomeres into chromatin complexes seems to follow a common theme. In most organisms the protein-coated telomeric tract is looped or folded into a more compact structure, but species may differ in the precise details of how this folding is achieved. t-loops may be used to stabilize folding in organisms that have long telomeres, but such structures have not yet been identified in organisms with shorter telomeres, so folding may be achieved solely as a result of protein-protein interactions. Folding of the telomere is thought to lead to a closed conformation that leaves the DNA terminus inaccessible to telomerase and other DNA-modifying activities. Destruction of t-loops and formation of an open, unfolded conformation is thought to occur during S phase, when the telomere must be accessible for replication.

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