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Telomeres Do D-Loop–T-Loop

Minireview

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Rarely it seems does a new result emerge, loop around, and bite you from behind. But a collaboration between Jack Griffith's and Titia de Lange's laboratories to look at the binding of telomere proteins to telomeric DNA has done just that. Griffith and de Lange show, in this issue of *Cell*, that rather than ending as linear DNA molecules as was previously thought, telomeres in mammalian cells end as large terminal loops (Griffith et al., 1999). Following the nomenclature of three-stranded DNA displacement loops, or D loops, and RNA displacement loops, or R loops, described by electron microscopy, they call these new structures "t loops" for telomere loops. The long stretches of the double-stranded telomere DNA are looped around, and the single-stranded terminus is tucked back inside the double-stranded DNA molecule—thus protecting the chromosome terminus (Figure 1). These results force us to rethink the classical view of telomere structure and function developed over the past 20 years and provide a context for a new synthesis.

The Classical View

Telomere Structure. Telomere structure is conserved in almost all eukaryotes. The DNA sequence at telomeres typically consists of tandem GT-rich repeats: (TTGGG G)n in *Tetrahymena*, (TTTTGGGG)n in a distantly related ciliate, *Oxytricha*, and (TTAGGG)n in human and mouse. After the initial identification of the simple sequence repeats at telomeres in *Tetrahymena* (Blackburn and Gall, 1978), Klobutcher et al. showed that in *Oxytricha*, the G strand is longer than the C strand, indicating a G strand 3′ end overhang at telomeres (Klobutcher et al., 1981). This provided the first evidence for the end structure that is conserved in all species examined to date. The size of the overhang is species specific, varying from an exact length of 16 nucleotides in *Oxytricha* to a variable stretch of 50–100 nucleotides in mouse and human telomeres. The fact that mutants that disrupt the G strand overhang also disrupt telomere function suggests that this overhang is important for telomere function (Garvik et al., 1995; Gravel et al., 1998; van Steensel et al., 1998).

Telomere Function. The primary role of telomeres is to protect chromosome ends from recombination, fusion, and from being recognized as damaged DNA. In addition they must allow access to the telomerase enzyme to add telomere repeats and maintain telomere length. In the absence of a functional telomere, a free DNA end is unstable and is subject to cellular processes that repair DNA breaks. Broken chromosomes signal DNA damage checkpoints; they are degraded by nucleases and participate in end-joining reactions that fuse two free ends (reviewed in de Lange, 1995). A major question in the telomere field is how telomeres provide stability and avoid detection as a broken DNA end. Evidence to date has suggested that telomere-binding proteins provide these functions.

Telomere-Binding Proteins. The binding proteins that mediate telomere function come in two flavors: double-stranded DNA-binding proteins and single-stranded DNA end–binding proteins. The best-characterized end-binding protein is the α/β heterodimer protein from *Oxytricha* (reviewed in Fang and Cech, 1995). This protein binds tightly to the single-stranded regions of the G strand overhang. Recently the crystal structure of this protein complexed with DNA was solved (Horvath et al., 1998). The single-stranded G-rich overhang is completely protected within the protein core and the 3′ end is inaccessible, as anticipated from biochemical experiments (Gottschling and Zakian, 1986; Froelich-Ammon

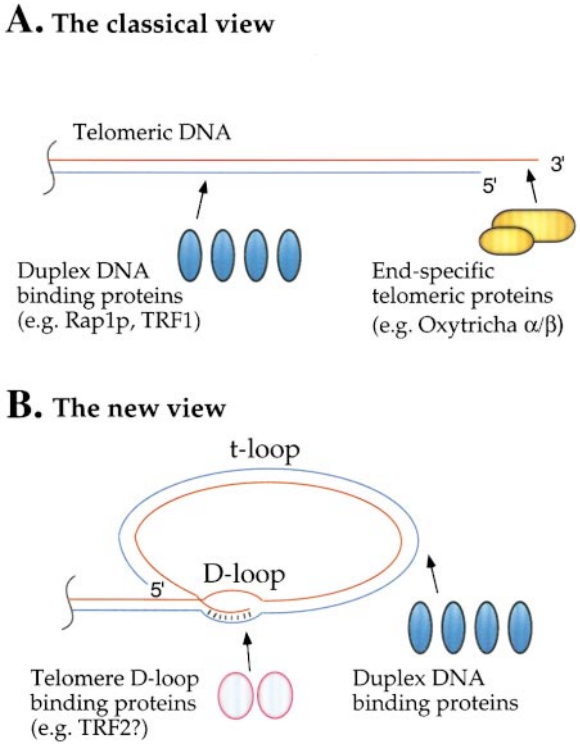

Figure 1. Classical versus New View of Telomere Structure

(A) The classical view of telomere structure. Linear double-stranded telomere repeats make up the bulk of the telomere sequence. Duplex telomere-binding proteins such as Rap1p (in yeast) and TRF1 (in mammalian cells) bind along the length of the telomere repeat tracts. At the end there is a 3′ overhang on the G strand. The end is thought to be bound by an end-specific telomere protein exemplified by the *Oxytricha* α/β telomere–binding protein.

(B) The new view of telomere structure. The telomere DNA loops back on itself forming a lariat structure. The 3′ G strand extension invades the duplex telomeric repeats and forms a D loop (displacement loop). Duplex DNA telomere–binding proteins bind along the length of the telomere repeats as above. A specialized telomere-binding protein binds the D loop at the junction of the lariat. TRF2 may play this role in stabilizing or allowing formation of the D loops.

et al., 1998). The other class of telomere-binding proteins binds double-stranded telomeric DNA. Examples are Rap1p from yeast and TRF1 from humans; both of these proteins bind along the length of the duplex telomeric repeat array. In both cases, the amount of the protein in the cell can affect telomere length: increasing protein levels by overexpression decreases telomere length, while less protein or dominant-negative versions result in longer telomeres (reviewed in Shore, 1997). Another telomere protein, TRF2, has been classified as a double-stranded telomere-binding protein. Curiously, however, dominant-negative TRF2 affects the single-stranded overhang at telomeres despite the fact that the protein binds only double-stranded TTAGGG/CCCTAA repeats in vitro. Overexpression of a dominant-negative version of TRF2 in cells results in loss of TRF2 bound at telomeres, loss of the G strand overhang, induction of the p53 damage pathway, and chromosome end fusions (van Steensel et al., 1998; Karlseder et al., 1999). How this protein, which binds the double-stranded telomere repeats, might affect the single-stranded tail at telomeres in vivo was not clear until the discovery of t loops.

T Loops

The Griffith and de Lange labs examined mammalian telomere structure by electron microscopy. Artificial telomeres generated in vitro were incubated with TRF2 and viewed in the electron microscope by the Kleinschmitt spreading technique. Unexpectedly, about 42% of the molecules had formed large loops in a reaction that depended on the presence of a 3′ G strand overhang at the end of the artificial telomere. This free 3′ end was tucked back inside the double-stranded DNA at the loop junction. TRF2 was bound at the base of the loop—presumably the site where the G strand invades the duplex DNA—suggesting that TRF2 mediates the formation of, or stabilization of, t loops (Figure 1B). Omission of TRF2 from the in vitro binding reaction resulted in a substantial reduction in the number of molecules found as loops from around 42% down to 2%–12%.

To determine whether t loops are present at telomeres in vivo, Griffith and de Lange devised a method to purify telomeric DNA from human cells in sufficient amounts for viewing in the EM. In addition, they employed psoralen cross-linking to stabilize t loops that might exist in vivo before removing proteins from the DNA. The fact that these two procedures had not been combined before may explain why t loops had not been previously documented. One would not expect to see the tiny fraction of telomere fragments in a preparation of total genomic DNA spread for EM because the telomeres would represent a vanishingly small proportion of the total DNA molecules. Analysis of the telomere fragments isolated from cells indicated that indeed t loops are present at telomeres in mammalian cells. Telomeric structures isolated from HeLa cells showed a large proportion of molecules (15%–40% in 15 different experiments) were preserved as t loops. Telomere DNA isolated from mouse liver also contained a high fraction of t loops. Only telomeric repeats were in the looped-out region and the loops were very large, often encompassing 10–20 kilobases of telomeric sequence. Cells where the chromosomes were know to have long telomeres contained larger loops than those that have short telomeres. This implies some mechanism that must regulate loop size. Although only a portion of the molecules were found as t loops in the EM experiment, given the technical difficulty of preserving this structure during sample preparation it is not yet clear whether all telomeres are actually in the t loop conformation throughout the cell cycle.

The observation of looped DNA in the EM might be an artifact of DNA folding back on itself during preparation. Several observations, however, argue against this possibility. First, the t loops are much larger than those formed from random flopping of DNA in EM preparations. The average size of DNA circles formed by random collision seen in EM is 300–500 base pairs (Howard et al., 1991). Second, a mixing experiment was done to examine the ability of telomere DNA to randomly flop back on itself under the conditions used to identify t loops. Uncross-linked DNA was isolated from cells with long telomeres and deproteinized. This DNA was mixed with nuclei from cells with short telomeres, the mixture was psoralen cross-linked, and telomere DNA was isolated. In these preparations, small circles were 10 times more abundant than large circles, arguing against the t loops being artifacts of the preparation method. Finally, the demonstration of a 100–200 base pair D loop (displacement loop) in which a single-stranded DNA invades a homologous double-stranded region and base pairs with one of the two strands, suggests the t loops are held in place by a specific structure and are not formed by random looping of the DNA in the EM preparation. In the D loop the TTAGGG G strand overhang of the telomere is base paired to the internal CCCTAA tracts, protecting the terminus and creating a structure that is distinct from a broken DNA end.

The model then is that t loops protect telomeres by physically stitching the potentially vulnerable single-stranded G strand terminus back into the double-stranded telomere sequence several kilobases internal to the terminus, forming a t loop. This interweaving appears to be mediated by TRF2 and results in a small D loop at the point of G strand insertion (Figure 1B). In the words of Jack Griffith, this means that telomeres are doing “D-loop–t-loop.” One important piece of evidence for this model that has not yet been examined in vivo is the role of TRF2 in mediating t loop formation. The fact that TRF2 bound the D loop junction in the in vitro experiments suggests that this protein mediates the formation of the D loop. This observation could explain the curious finding that overexpression of a dominant-negative version of this double-stranded telomere-binding protein results in loss of the G strand overhang (van Steensel et al., 1998). A prediction of the current model is that overexpression of dominant-negative TRF2 in vivo will result in loss of the t loops. This additional control will also address concerns over the possible artifactual looping of DNA in the EM. If a cell line expressing dominant-negative TRF2 has no t loops and the sister cell line without dominant-negative TRF2 does have them, this would further support the functional role of these structures.

The New Synthesis

The discovery of t loops at mammalian telomeres necessitates a reevaluation of the mechanisms that mediate telomere function. The discovery of t loops solves some long-standing problems, makes others moot, and raises

new ones. Models for both telomere elongation and telomere silencing have mostly drawn telomeres in a linear configuration. Models are powerful tools in molecular biology: they focus our thinking and allow the design of specific experiments. They also, however, can limit our thinking when important aspects of a system are not represented.

Telomere-Binding Proteins. The classic telomere end-binding protein from *Oxytricha* has been thought to provide the protective function at telomeres. But perhaps *Oxytricha* in this instance is the outlier rather than the paradigm. The discovery of t loops now suggests that telomere protection can be mediated by sequestering the 3′ G strand overhang inside a double-stranded DNA rather than by tenacious protein binding. Interestingly, a telomere end–binding protein similar to that of *Oxytricha* has not been isolated from any other species, although a protein with similar properties has been characterized in *Xenopus* (Cardenas et al., 1993). *Oxytricha* telomeres consist of just of 20 bp of double-stranded (TTTTGGGG/CCCCTTTT—far too short to form t loops. Perhaps in species with longer telomeres, the t loop structure substitutes for the end-binding protein to protect the end. Although this model is consistent with current knowledge, the lack of detection of end-binding proteins in other species might be simply a technical one. One needs to be cautious in suggesting that ciliates solve biological problems in unusual ways since historically many important processes that are widely conserved in eukaryotes, such as telomere sequences, catalytic RNA, telomerase and histone acetyltransferase were first discovered in ciliates (Blackburn and Gall, 1978; Kruger et al., 1982; Greider and Blackburn, 1985; Brownell et al., 1996).

If t loops are indeed essential features of telomere function, they would be expected to be phylogenetically conserved. It will be of particular interest to determine if t loops are at telomeres in yeast, where telomere length regulation has been well characterized. Several features of yeast telomeres make it less likely that t loops will form there. First, they are significantly shorter than mammalian telomeres, containing about 300–400 base pairs of repeated sequence. This is likely long enough to form t loops, but just barely (Howard et al., 1991). The fact that the telomere-binding protein Rap1p will bend DNA may aid in t loop formation (reviewed in Fang and Cech, 1995). Also the irregular sequence that makes up the yeast telomere sequence provides many fewer places where a stable D loop could form. However, as in human cells, a tenacious end-binding protein has not yet been identified in yeast. The best candidate for an end-binding protein is Cdc13p; yet the binding of this protein to does not have the same end specificity as the *Oxytricha* end-binding protein (Garvik et al., 1995; Lin and Zakian, 1996; Nugent et al., 1996). Thus, given the structure of yeast telomeres, it is the ideal test case to determine if t loops are phylogenetically conserved. If telomere function mediated by t loops is conserved, models for the role of telomere binding proteins in telomere function will need to be reexamined.

Telomere Function. T loops can explain how the very tip of a chromosome is distinguished from a DNA break. Presumably the machinery that recognizes DNA breaks requires access to the very end. Thus, the sequestration of the G tail in the t loop may prevent an inappropriate DNA damage response. The t loops are also likely to mediate the stability function of telomeres either directly or indirectly. The DNA damage response pathway itself may be responsible for G strand degradation and end-to-end fusion of chromosomes (Karlseder et al., 1999). Thus, blocking recognition by this checkpoint pathway may be sufficient to stabilize chromosome ends.

A second essential function of telomeres is to allow access to telomerase and to establish a telomere length equilibrium. The discovery of t loops creates a new question for telomerologists to solve. How does telomerase access the now hidden 3′ terminus to elongate the telomere? In previous models where telomeres are tightly bound by a protein, the problem was that the protein had to be removed for telomerase to access and elongate the G strand. Now, with t loops, the base-paired D loop structure must be unwound, perhaps by a specific helicase, to allow access by telomerase. This suggests that the t loop structure must be dynamic and reform after each round of DNA synthesis. The unique structure of the t loop, however, might allow easy recognition by telomerase as the appropriate substrate for elongation.

Telomere Structure. The telomere shortening and subsequent loss of function observed in cells that lack telomerase may be due to disruption of the t loop structure. In several organisms in which telomere shortening is seen, telomeres lose the ability to protect against chromosome fusion well before all telomere sequence is lost. Perhaps a minimal length is needed to form a t loop, and once telomeres are sufficiently short, they can no longer protect the end. The minimal length for t loop formation may also be species specific, as are both double-stranded telomere tracts and G strand overhang length. This model can be tested in vivo by looking at senescent human cells as well as telomerase-null mouse and yeast cells. Does the proportion of t loops decline as the telomeres shorten and lose function?

Much remains to be learned about the formation and regulation of t loops. The earliest characterization of telomere structure and function involved cytological observations of broken chromosomes by Muller and McClintock in the late 1930s (reviewed in Gall, 1995). After the identification of telomere DNA sequences in the late 1970s, the telomere field went molecular. In the past 20 years we have learned much about telomere synthesis as well as how biochemical interactions of DNA and telomere proteins regulate telomere length. But now, 60 years after the experiments of Muller and McClintock, cytology has quietly snuck up from behind and made us take notice. The simple visualization of telomeres has again given us a new view of telomere structure and function.

Selected Reading

Blackburn, E.H., and Gall, J.G. (1978). J. Mol. Biol. *120*, 33–53.

Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D.G., Roth, S.Y., and Allis, C.D. (1996). Cell *84*, 843–851.

Cardenas, M.E., Bianchi, A., and de Lange, T. (1993). Genes Dev *7*, 883–894.

de Lange, T. (1995). In Telomeres, E.H. Blackburn and C.W. Greider, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 265–293.

Fang, G., and Cech, T.R. (1995). In Telomeres, E.H. Blackburn and C.W. Greider, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 69–105.

Froelich-Ammon, S.J., Dickinson, B.A., Bevilacqua, J.M., Schultz, S.C., and Cech, T.R. (1998). Genes Dev. *12*, 1504–1514.

Gall, J.G. (1995). In Telomeres, E.H. Blackburn and C.W. Greider, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 1–10.

Garvik, B., Carson, M., and Hartwell, L. (1995). Mol. Cell. Biol. *15*, 6128–6138.

Gottschling, D.E., and Zakian, V.A. (1986). Cell *47*, 195–205.

Gravel, S., Larrivee, M., Labrecque, P., and Wellinger, R.J. (1998). Science *280*, 741–744.

Greider, C.W., and Blackburn, E.H. (1985). Cell *43*, 405–413.

Griffith, J.D., Comeau, L., Rosenfield, S., Stansel, R.M., Bianchi, A., Moss, H., and de Lange, T. (1999). Cell *97*, this issue, 503–514.

Horvath, M.P., Schweiker, V.L., Bevilacqua, J.M., Ruggles, J.A., and Schultz, S.C. (1998). Cell *95*, 963–674.

Howard, M.T., Lee, M.P., Hsieh, T.S., and Griffith, J.D. (1991). J. Mol. Biol. *217*, 53–62.

Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., and de Lange, T. (1999). Science *283*, 1321–1325.

Klobutcher, L.A., Swanton, M.T., Donini, P., and Prescott, D.M. (1981). Proc. Natl. Acad. Sci. USA *78*, 3015–3019.

Kruger, K., Grabowski, P.J., Zaug, A.J., Sands, J., Gottschling, D.E., and Cech, T.R. (1982). Cell *31*, 147–157.

Lin, J.J., and Zakian, V.A. (1996). Proc. Natl. Acad. Sci. USA *93*, 13760–13765.

Nugent, C.I., Hughes, T.R., Lue, N.F., and Lundblad, V. (1996). Science *274*, 249–252.

Shore, D. (1997). Trends Biochem. Sci. *22*, 233–235.

van Steensel, B., Smogorzewska, A., and de Lange, T. (1998). Cell *92*, 401–413.