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T-loop assembly *in vitro* involves binding of TRF2 near the 3′ telomeric overhang

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Mammalian telomeres contain a duplex TTAGGG-repeat tract terminating in a 3′ single-stranded overhang. TRF2 protein has been implicated in remodeling telomeres into duplex lariats, termed t-loops, *in vitro* and t-loops have been isolated from cells *in vivo*. To examine the features of the telomeric DNA essential for TRF2-promoted looping, model templates containing a 500 bp double-stranded TTAGGG tract and ending in different single-stranded overhangs were constructed. As assayed by electron microscopy, looped molecules containing most of the telomeric tract are observed with TRF2 at the loop junction. A TTAGGG-3′ overhang of at least six nucleotides is required for loop formation. Termini with 5′ overhangs, blunt ends or 3′ termini with non-telomeric sequences at the junction are deficient in loop formation. Addition of non-telomeric sequences to the distal portion of a 3′ overhang beginning with TTAGGG repeats only modestly diminishes looping. TRF2 preferentially localizes to the junction between the duplex repeats and the single-stranded overhang. Based on these findings we suggest a model for the mechanism by which TRF2 remodels telomeres into t-loops.

Keywords: electron microscopy/telomere/t-loop/TRF2

Introduction

Telomeres are specialized nucleoprotein structures at the ends of linear chromosomes comprised of repeated DNA elements and specific DNA binding proteins. In mammals, the duplex hexameric repeat TTAGGG runs 5′–3′ toward the chromosome end and terminates in a 75–300 nucleotide (nt) single-stranded (ss) 3′ overhang of the G-rich strand (Makarov *et al*., 1997; McElligott and Wellinger, 1997; Wright *et al*., 1999). The length of the repeat tract, while variable, is organism specific, with human telomeres ranging from 5 to 30 kb while mouse telomeres can be long as 150 kb (Moyzis *et al*., 1988; de Lange *et al*., 1990; Kipling and Cooke, 1990; Lejnine *et al*., 1995). The machinery that reproduces the genome replicates the bulk of the telomere. During each round of replication, telomeres shorten by ~70–200 bp, due in part to the 'end replication problem' (Watson, 1972; Olovnikov, 1973; Harley *et al*., 1990). The reverse transcriptase telomerase restores the lost sequences in germ line and transformed cells (Greider and Blackburn, 1985; Lingner *et al*., 1997; reviewed in Oulton and Harrington, 2000).

Two unique mammalian proteins, TRF1 and TRF2, have been discovered, which bind exclusively to double-stranded (ds) telomeric DNA. Both form homo- but not hetero-dimers (Chong *et al*., 1995; Bilaud *et al*., 1997; Broccoli *et al*., 1997) and bind DNA through two Myb domains, one at the C-terminus of each monomer. TRF1 and TRF2 differ in their N-termini, which are rich in either acidic residues (TRF1) or basic residues (TRF2). *In vivo*, both proteins localize to the telomere throughout the cell cycle (Chong *et al*., 1995; Luderus *et al*., 1996; Broccoli *et al*., 1997). 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 (van Steensel and de Lange, 1997; Smogorzewska *et al*., 2000). In addition to TRF1 and TRF2, mammalian telomeric DNA *in vivo* is complexed by a host of cellular proteins, including the histones (Makarov *et al*., 1993; Tommerup *et al*., 1994; Cacchione *et al*., 1997), DNA repair factors such as Ku (Hsu *et al*., 1999) and the Mre11–Rad50–NBS1 complex (Zhu *et al*., 2000). Tin2 (Kim *et al*., 1999), hRap1 (Li *et al*., 2000) and tankyrase (Smith *et al*., 1998) associate indirectly with telomeric DNA via binding to TRF1 or TRF2.

Human cells must distinguish their 92 chromosome ends from internal ds breaks, which, if present at this number, would trigger cell cycle arrest and apoptosis. TRF2 plays a central role in concealing telomere ends from ds break recognition and repair factors. Expression of a dominant-negative allele of TRF2 in cultured human cells rapidly triggers changes typical of those induced by ds breaks: induction of apoptosis through the ATM/p53-dependent DNA damage checkpoint pathway (Karlseder *et al*., 1999), loss of the 3′ ss overhang and induction of end-to-end chromosome fusions (van Steensel *et al*., 1998).

Our recent study of mammalian telomeres provided a possible structural solution to how telomere ends are sequestered from the DNA repair pathways. This study showed that mammalian telomeres are arranged into large duplex loops *in vivo* (t-loops) (Griffith *et al*., 1999). It was proposed that the t-loop is formed by strand invasion of the 3′ overhang into the preceding telomeric tract to form a lariat with a D-loop at the loop–tail junction and that this would effectively hide the natural end of the DNA to protect it from the machinery that scans DNA for broken ends. Recently, t-loops were found at the termini of micronuclear chromosomes of *Oxytricha nova* (Murti and Prescott, 1999) and at the telomeres of *Trypanosome brucei* minichromosomes (Munoz-Jordan *et al*., 2001). Furthermore, telomeres in *Saccharomyces cerevisiae*

appear to form fold-back structures (Grunstein, 1997; de Bruin *et al*., 2000, 2001). Thus, telomere looping may be a common theme in telomere architecture.

In our previous study, we generated a model telomere DNA containing ~2 kb of ds TTAGGG repeats at the end of a linearized plasmid DNA and terminating in a 3′ ss overhang. Incubation of this DNA with TRF2, and inspection of the resulting complexes by electron microscopy (EM), revealed that TRF2 was able to either catalyze t-loop formation or stabilize loops formed by the ss tract folding back to associate with the internal duplex repeats. TRF2 localized exclusively to the loop junction (Griffith *et al*., 1999). Neither TRF1 nor tankyrase arranged the DNA into loops and a 3′ G-strand overhang was required for loop formation: DNA molecules with 5′ overhangs or blunt ends were inefficient as templates. Cross-linking data supported a model in which the 3′ overhang invades the telomeric duplex to form a D-loop. It was unclear, however, how TRF2 formed these loops since it does not bind ss telomeric DNA (Broccoli *et al*., 1997; T.de Lange, A.Bianchi, R.M.Stansel and J.D.Griffith, unpublished results).

To begin to understand how TRF2 promotes looping, we have generated a model telomere DNA in which the ss overhang can be altered in sequence, length and orientation (Figure 1A). Using these variants we examined the ability of TRF2 to bind and to induce t-loop formation with each variant. The results point to the critical importance of the natural ss/ds junction at the 3′ telomeric overhang in positioning TRF2 on the DNA and inducing t-loop formation. Possible models for how this interaction initiates t-loop formation are discussed.

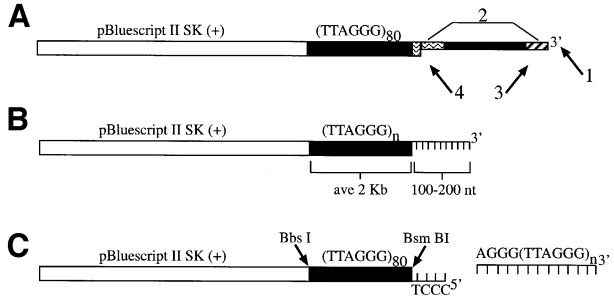

Fig. 1. Model telomere templates and the variants of the model telomere$_{500bp}$ termini. The model telomere$_{500bp}$ termini were altered in the orientation of the overhang (1), the length of the overhang (2), the sequence of the 3′-end of the overhang (3) and the sequence of the ss/ds junction (4) (**A**). A telomere-containing clone was engineered to place a nine repeat telomere tract at the end of the linearized plasmid. Long telomeric duplexes were created from this clone by both unidirectional replication (model telomere$_{\pm 2kb}$) (**B**) and expansive cloning (model telomere$_{500bp}$) (**C**). Unidirectional replication (B) utilizes a single telomeric repeat containing oligonucleotide to extend the duplex tract off the end of the linearized plasmid. The result is a wide range of tract sizes (average 2 kb). To generate the 3′ overhang, the model telomere$_{\pm 2kb}$ DNA was digested with a 5′–3′ exonuclease. Expansive cloning (C) utilizes continued cycles of cloning a nine repeat insert to generate long tracts of fixed lengths. The longest stable tract achieved is ~500 bp. The model telomere$_{500bp}$ template, when linearized, contains a 4 nt 5′ overhang to which an oligonucleotide can be ligated to create a variety of model DNAs (A).

Results

Generation of model telomere DNAs

A model mammalian telomere DNA should begin with non-telomeric sequences, be followed by a long tract of ds 5′-TTAGGG-3′/3′-AATCCC-5′ repeats, and terminate in a 3′ ss overhang of the G-rich strand. Previously (Griffith *et al*., 1999), a model template was generated by unidirectional replication, resulting in an average TTAGGG tract length of 2 kb ± 800 bp with some tracts as long as 5 kb, in the range of human telomere lengths *in vivo* (termed here model telomere$_{\pm 2kb}$; see Materials and methods) (Figure 1B). Overhangs (3′) were generated using T4 gene 6 exonuclease. To provide a template in which the overhang and ss/ds junction could be manipulated, a model DNA with a fixed number of TTAGGG repeats (model telomere$_{500bp}$) was created by expansive cloning (see Materials and methods) (Figure 1C). The DNA was engineered such that long oligonucleotide tails can be added in either the 5′ or 3′ orientations. To monitor the efficiency of adding the overhang, two biotin moieties were incorporated into each oligonucleotide. Following ligation and incubation with streptavidin, examination by EM revealed that ~80% of the model telomere$_{500bp}$ DNA molecules contained an overhang. This test was carried out for each DNA construct described below. The DNAs were used only if at least 80% of the molecules contained tails. Following optimization, several of the tails were obtained without biotin to test the influence of this moiety on TRF2 binding and t-loop formation (no effect detected). Four features of the telomere end were examined with respect to looping (Figure 1A): (i) the orientation and sequence of the overhang (3′ or 5′ and G strand or C strand); (ii) the length of the 3′ overhang; (iii) the sequence of the distal portion of the 3′ overhang; and (iv) the ss sequence at the junction.

T-loops form efficiently with natural 3′ termini

The template generated as a standard for these studies contains a 54 nt 3′ overhang, $(TTAGGG)_9$, added to the terminus of the model telomere$_{500bp}$ DNA. This substrate was incubated with TRF2 using conditions optimized by EM (three TRF2 dimers per repeat, 20 mM HEPES pH 8.75, 20 min; see Materials and methods) and mounted directly onto EM supports without fixation. At this ratio of TRF2 dimers to DNA molecules, either the DNA was protein free or showed only one TRF2 complex bound. The DNA was present in a variety of forms. Frequently, one DNA end was observed folded back into a loop of ~200–500 bp with TRF2 at the loop junction; structures we term t-loops. An example of a molecule containing a t-loop is shown in Figure 2A and enlargements of several loops are presented in Figure 3A–D. In addition, TRF2 was observed bound both internally along the DNA, but within 500 bp of the nearest end and thus presumably along the TTAGGG repeat tract (Figure 2B), and at one end of the DNA (Figure 2C). No DNAs were observed that had TRF2 bound both internally and at the end. Finally, linear DNA molecules with no protein bound were abundant (not shown) and aggregates of two or more DNAs bound by a large mass of TRF2 were present. In the case of aggregates involving only two DNAs, the DNA molecules appeared fused together at their ends (Figure 2D). Aggregates were observed with all of the templates and the level of aggregation increased as the ratios of TRF2 to DNA

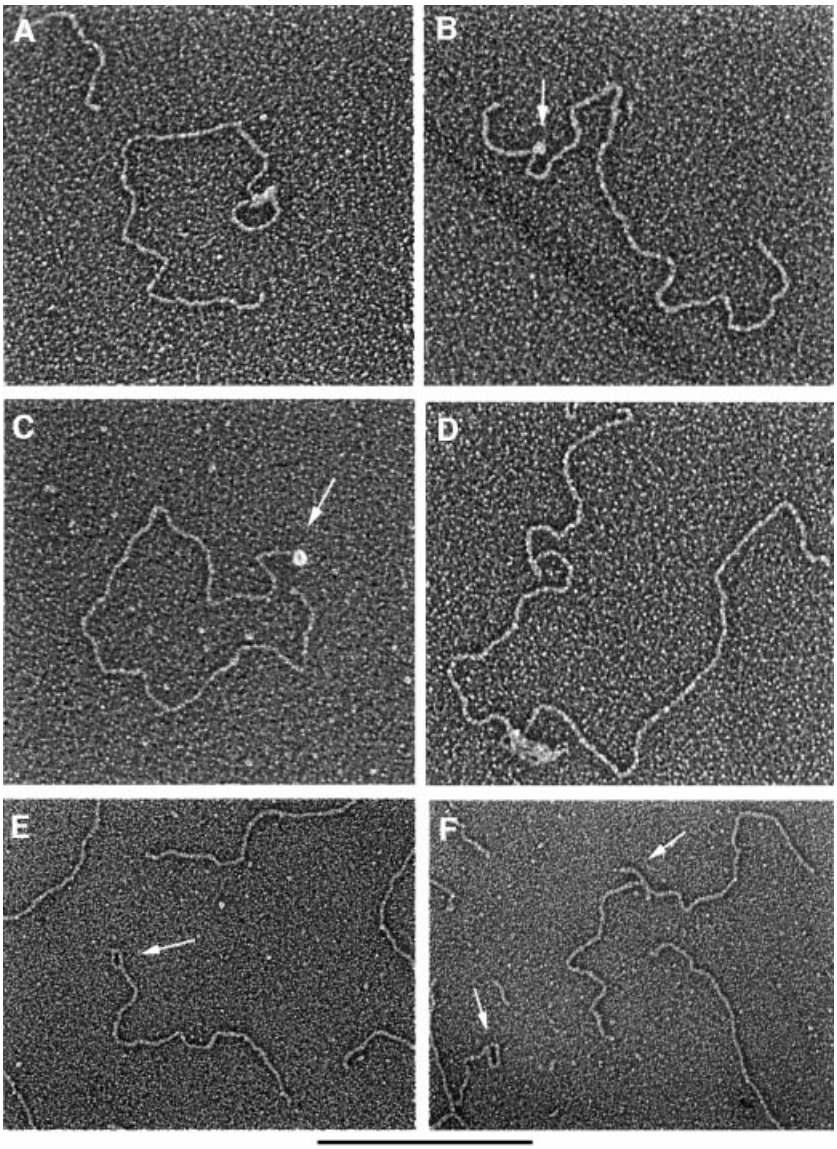

Fig. 2. Visualization of TRF2 binding to a model telomere template *in vitro*. (A–D) The model telomere$_{500bp}$ DNA with a 54 nt TTAGGG-3′ overhang was incubated with human TRF2 produced in insect cells under conditions described in the text and then directly adsorbed to the EM supports followed by washing, air-drying and rotary shadowcasting with tungsten. Molecules arranged into loops (**A**), with TRF2 bound internally on the 500 bp TTAGGG tract (**B**) or at one end of the DNA (**C**) were observed. In addition, synapsis between the ends of two molecules (**D**) were present. Following incubation with TRF2, aliquots of the sample were treated with psoralen and UV, followed by deproteinization, surface spreading with cytochrome *c* and rotary shadowcasting with platinum–palladium. Molecules with small loops at one end as well as two DNAs attached at their ends were present (arrows, **E** and **F**). Shown in reverse contrast. Bar is equivalent to the length of a 1.0 kb DNA.

increased. Since the number of DNA molecules contained within the aggregates could not be determined by EM and the level of aggregation appeared consistent between experiments (when the same level of TRF2 was used), they were not included in the total number of DNAs counted. Thus, the data below are presented as the percentage of the individual (non-aggregated) DNAs. A non-biotin-labeled 54 nt overhang was also prepared and the same results were obtained, showing that the presence of a biotin tag on the tail does not affect the binding of TRF2 or its ability to form t-loops.

Using these scoring criteria, when the template with a 54 nt $(TTAGGG)_9$ 3′ overhang was incubated with TRF2 as indicated above and 1000 non-aggregated DNAs scored in 10 different experiments, 19 ± 8% had one end folded back into a loop with TRF2 at the loop junction, 32 ± 19% had a TRF2 particle bound at one end of the DNA (presumably the end with the telomeric repeats), 11 ± 5% had a TRF2 particle bound along the telomeric repeat tract and the remainder (39%) were scored as being protein free (Table I). Only looped forms that contained a protein complex at the loop junction were scored as t-loops. No TRF2 binding was observed with the parent plasmid lacking TTAGGG repeats. When the model telomere template was incubated with equivalent amounts of TRF1 (0.5–4 dimers per repeat) under conditions optimal for TRF1 binding (Griffith *et al*., 1998), TRF1 was observed bound to the repeat tract as clusters of protein balls (Figure 3E) or separate particles (Figure 3F), but with no looping (0%; n = 100/sample, two experiments).

Using these EM preparative methods, a single 120 kDa TRF2 dimer can be clearly distinguished bound to DNA

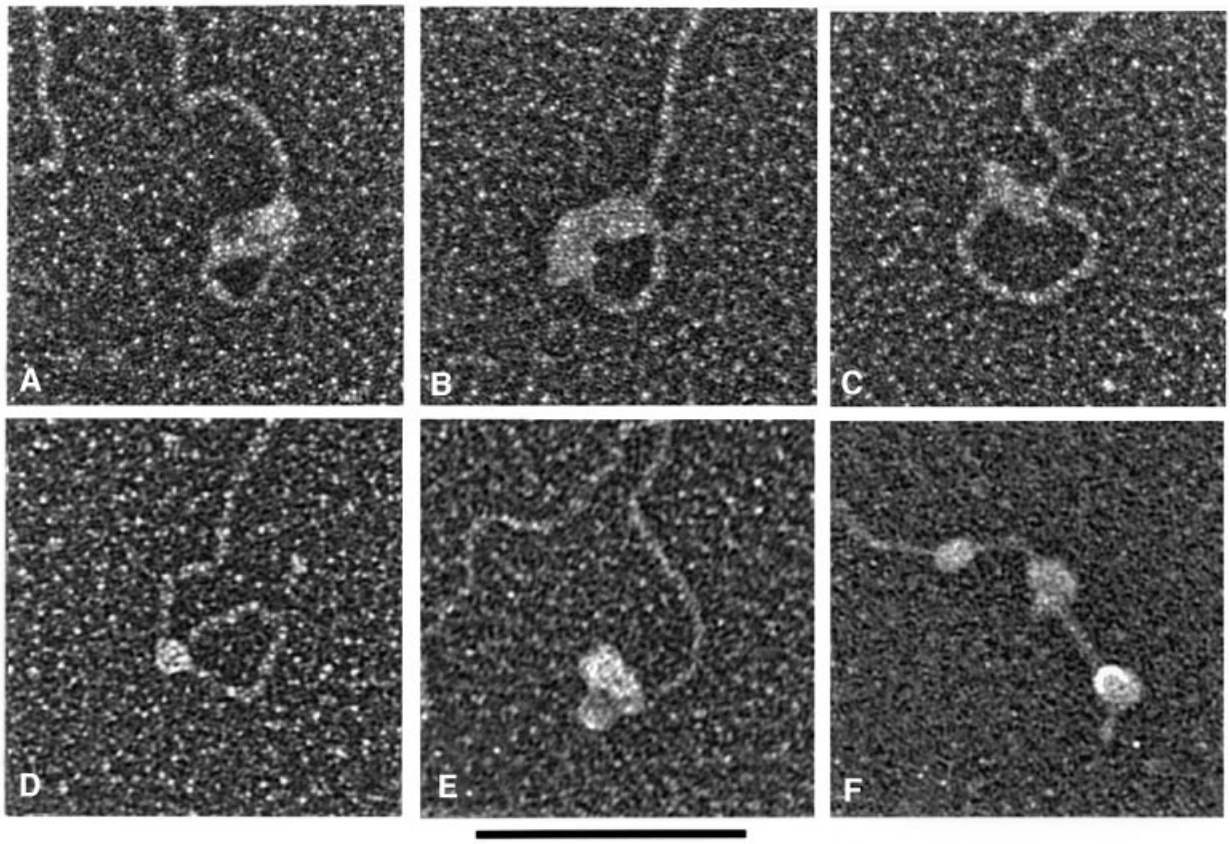

Fig. 3. Visualization of the ends of the model telomere DNA bound by TRF1 and TRF2. Examples of looped DNA molecules generated on the model telomere$_{500bp}$ DNA with a 54 nt TTAGGG-3′ overhang as described in Figure 2A–C shown at higher magnifications reveal a large oligomeric mass of TRF2 at the loop junction (**A**–**D**). Incubation of the same DNA with TRF1 (see text for details) generated DNA molecules with balls or chains of balls at one end (**E** and **F**). Shown in reverse contrast. Bar is equivalent to the length of a 500 bp DNA.

and hence we are confident of our ability to visually sort the DNA molecules into the different classes described above. The smallest mass of TRF2 observed at the t-loop junction was ~3 dimers, with the average mass being ~10 dimers. These estimates are based on experiments in which the complexes are mounted for EM in the presence of similar sized protein molecules of known size (Griffith *et al*., 1995). The TRF2 particles observed at the telomere end were most likely located at or adjacent to the ss/ds junction since TRF2 has a very low affinity for ss telomeric DNA in the presence of ds telomeric DNA (Broccoli *et al*., 1997; A.Bianchi, T.de Lange, R.M.Stansel, and J.D.Griffith, unpublished data). In the examples shown in Figures 2C and 3D, the particles are of a size suggestive of TRF2 tetramers, while the particle in Figure 2B is smaller, possibly having a mass of a single TRF2 dimer. The TRF2 masses shown bound to the loops in Figure 3A–C are clearly higher oligomeric forms.

The length of the loops formed by TRF2 using the model telomere$_{\pm 2kb}$ DNA measured 1800 ± 700 bp, while those formed on the model telomere$_{500bp}$ DNA measured 338 ± 190 bp; both close to the length of the telomere tracts in the templates (2000 ± 800 and 500 bp, respectively). If DNA flexibility alone had determined the size of the loops, then it would have been expected that both distributions would have been the same, and would have reflected the Shore and Baldwin value for optimal DNA circularization, which is ~200 bp (Shore *et al*., 1981).

A 3′ overhang with a single TTAGGG repeat is sufficient for t-loop formation

Since we previously reported that blunt-ended DNA does not provide a suitable template for t-loop formation (Griffith *et al*., 1999), we examined the ability of TRF2 to generate t-loops on DNAs with 3′-TTAGGG overhangs varying from 1 to 14 repeats. The DNAs were incubated with TRF2, and the number of t-loops and the location of the protein bound along the DNA were scored by EM (Table I). For templates with tails of 1, 2, 4, 9 and 14 repeats, loops were seen at frequencies of 11 ± 9%, 16 ± 8%, 14 ± 4%, 19 ± 8% and 12 ± 5% (n = 100/sample, four experiments). Thus, while looping required a homologous 3′ tail, it was not significantly enhanced by extending the tail beyond one repeat. Analysis of the binding of TRF2 to these DNAs showed that the level of binding remained highest at the extreme end of the telomeric DNA, regardless of the length of the overhang (Table I).

In our previous study employing the model telomere$_{\pm 2kb}$ DNA (Griffith *et al*., 1999), we showed that the t-loop junctions could be photocross-linked with

Table I. Effect of the single-stranded overhang on the number of t-loops and the frequency and location of TRF2 binding

500 bp [TTAGGG]n / plasmid — Terminal structure	total n	% unbound	% TRF2 internal	% TRF2 at end	% t-loops
---- TTAGGGTT>AGGG[TTAGGG]$_9$ 3' ---- AATCCCAATCCC	1000	39 (25)	11 (5)	32 (19)	19 (8)
---- TTAGGGTT 3' ---- AATCCCAA 5'	200	77 (10)	17 (4)	4 (4)	2 (2)
---- TTAGGGTT>AGGGTTAGGG ---- AATCCCAATCCC<AATCCC[AATCCC]$_9$ 5'	200	68 (1)	13 (<1)	16 (<1)	3 (1)
---- TTAGGGTT>AGGG[CCCTAA]$_9$ 3' ---- AATCCCAATCCC	300	74 (10)	22 (5)	4 (5)	1 (1)
---- TTAGGGTT>AGGG[TTGGGG]$_9$ 3' ---- AATCCCAATCCC	200	58 (2)	n/s	n/s	1 (1)
---- TTAGGGTT>AGGG 54 nt randomT/A/G 3' ---- AATCCCAATCCC	200	52 (3)	n/s	n/s	1 (<1)
---- TTAGGGTT>AGGG[AATCCC]$_9$ 3' ---- AATCCCAATCCC	300	50 (5)	30 (3)	16 (3)	3 (<1)
---- TTAGGGTT>AGGGTTAGGG 3' ---- AATCCCAATCCC	400	60 (25)	13 (7)	18 (12)	11 (9)
---- TTAGGGTT>AGGG[TTAGGG]$_2$ 3' ---- AATCCCAATCCC	400	32 (10)	12 (3)	41 (5)	16 (8)
---- TTAGGGTT>AGGG[TTAGGG]$_4$ 3' ---- AATCCCAATCCC	400	46 (18)	9 (5)	33 (21)	14 (4)
---- TTAGGGTT>AGGG[TTAGGG]$_{14}$ 3' ---- AATCCCAATCCC	400	51 (15)	10 (5)	27 (7)	12 (5)
---- TTAGGGTT>AGGGTTAGGGN$_{48}$ 3' ---- AATCCCAATCCC	400	54 (9)	24 (3)	8 (3)	14 (6)
---- TTAGGGTT>AGGG[TTAGGG]$_2$N$_{42}$ 3' ---- AATCCCAATCCC	400	49 (19)	25 (11)	11 (4)	15 (8)
---- TTAGGGTT>AGGG[TTAGGG]$_8$N$_{10}$ 3' ---- AATCCCAATCCC	300	49 (36)	19 (15)	24 (17)	8 (5)
---- TTAGGGTT>AGGGN$_6$[TTAGGG]$_8$ 3' ---- AATCCCAATCCC	300	52 (10)	40 (17)	7 (6)	1 (1)

The '>' signs in the terminal structures represent the ligation site of the oligonucleotide overhangs. The percentages of the molecules unbound, internally bound, end bound and looped molecules are expressed as a fraction of the total molecules. The numbers in parentheses represent the standard deviation from one experiment to another (n = 100 per experiment).

the psoralen derivative 4′aminomethyl trioxalen (AMT) and UV light. The covalent cross-links fixed the looped structures in place, preserving them so that when the DNA was spread on an air–water interface with a denatured protein film (Kleinschmidt and Zahn, 1959) looped DNA molecules were observed. This was interpreted as evidence that the t-loop junction involves base pairing of the ss overhang with a segment of the ds TTAGGG repeat tract.

Psoralen photocross-linking was performed following incubation of TRF2 with the model telomere$_{500bp}$ templates containing 3′ overhangs of 1, 2, 4, 9 and 14 repeats, and with the DNA containing nine repeats but with no TRF2 in the incubation. In the presence of TRF2 (Figure 2E and F), DNA molecules with loops at one end were observed at frequencies of 9 ± 1%, 11.5 ± 1.5%, 11 ± 3%, 13 ± 2% and 12 ± 2% (n = 100/sample, three experiments) for tails of 1, 2, 4, 9 and 14 repeats, respectively. In the sample with no TRF2, 4 ± 1% of the DNA molecules (n = 100/sample, three experiments) were scored as having a loop at one end, most likely reflecting accidental juxtapositioning. In the incubations with TRF2, synapsis between the ends of two DNA molecules was occasionally observed (Figure 2F), suggesting that the forms shown in Figure 2D had been cross-linked. Since the TRF2 was removed prior to spreading the DNA for EM, we were unable to eliminate molecules in which the end has bent back around near an internal segment but in which there was no protein at the junction.

Under the reaction conditions used here, AMT/UV treatment generates cross-links roughly every 100 bp (Griffith *et al*., 1999). Since the t-loops formed on the DNAs with short (<54 nt) overhangs were stabilized by this treatment, it appears that more than just the nucleotides of the ss tail were inserted into the duplex to form the D-loop. This could result from TRF2-induced unwinding of the duplex DNA at the ss/ds

junction, allowing segments of both the G- and C-rich strands to pair in the t-loop junction and assist in t-loop stabilization.

T-loop formation and TRF2 end binding are greatly reduced by non-telomeric sequences at the ss/ds junction

A series of constructs were generated using the model telomere$_{500bp}$ DNA to examine the dependence of looping and protein binding on the nature of the ss/ds telomere junction. TRF2-dependent looping was not observed at significant frequencies when the DNA terminated in a blunt end (2 ± 2%; n = 100/sample, two experiments) (Table I). Three templates were then engineered in which the overhang was not homologous to the duplex telomeric tract. One contained 54 nt of a $(TTGGGG)_9$ 3′ repeat, while the ss 3′ tail for the second was 54 nt of a random sequence with the same ratio of T, A and G residues (2:1:3) as telomeric DNA. As scored by EM, neither template formed t-loops (1 ± 1%; n = 100/sample, two experiments) (Table I), demonstrating that homology between the duplex and tail is essential for t-loop formation. The third variant was one in which the DNA terminated in a 54 nt $(AATCCC)_9$ 3′ overhang. Were this overhang to pair with the G-rich strand, there would be two mismatches per repeat. T-loops were not assembled efficiently on this DNA either (3 ± 0%, n = 100/sample, three experiments).

A template containing homology to the C strand, consisting of a CCCTAA 5′ overhang, also failed to form a significant number of loops (3 ± 1%; n = 100/sample, two experiments) (Table I). To determine whether this was due to the 5′ orientation or sequence, an oligonucleotide consisting of 54 nt of the C-rich repeat $(CCCTAA)_9$ was ligated to the model template in the 5′–3′ orientation (Table I). This overhang is capable of base pairing within the duplex, but the end would terminate facing the centromeric portion of the telomere, as opposed to the standard t-loop junction, which terminates facing the terminus of the telomere. T-loops were not formed at a significant frequency with this DNA (1.5 ± 1%; n = 100/sample, three experiments) and cross-linking studies verified the lack of t-loop formation by TRF2 on this template (2 ± 2%; n = 100/sample, four experiments).

To examine the importance of homology at the ss/ds junction, a template was prepared in which the first 6 nt at the beginning of the tail (nearest to the ss/ds junction) were non-telomeric, followed by eight natural TTAGGG-3′ repeats [$N_6(TTAGGG)_8$ 3′] (Table I). When this DNA was incubated with TRF2, almost no loops were observed (1 ± 1%; n = 100/sample, three experiments).

Examination of the pattern of TRF2 binding to these different DNA molecules showed that the localization of TRF2 to the extreme end of the telomeric tract, while variable, was reduced ~5-fold relative to the DNA with a 54 nt 3′ natural overhang (Table I). Thus, although TRF2 does not bind to ssDNA directly, alteration of the sequence of the overhang proximal to the ss/ds junction affects the localization of TRF2 to the end of the DNA, suggesting some ability to respond to the ss sequence. In contrast, binding at internal sites was not influenced by the structure of the terminus and there was no significant difference in overall affinity of TRF2 for the DNAs with different terminal structures. Binding to the end demonstrated a stronger correlation with the ability of TRF2 to remodel the DNAs into t-loops (r = 0.67) than was found for binding of TRF2 to internal sites (r = 0.51) (p = 0.0035). These results suggest that the structure and sequence of the end of the DNA are important in positioning TRF2 onto the telomeric DNA, presumably a crucial step in t-loop formation. Alteration of the ss portion of the junction also may reduce the ability of TRF2 to initiate melting of the duplex, which would inhibit strand invasion and result in reduced t-loop formation. Given the higher representation of the internal sites, it is likely that much of the TRF2 would initially bind internally and perhaps translocate to a terminal position.

A construct was prepared by linearizing the model telomere$_{500bp}$ to place the duplex repeats in the center of the molecule. A single-stranded $(TTAGGG)_9$ overhang was then ligated to the template to produce an 'interrupted' model telomere DNA with 1.5 kb separating the overhang from the TTAGGG repeat tract. In this substrate, the duplex portion of the junction was not telomeric. Inspection of the products following incubation with TRF2 showed no significant loops formed between the end and the internal repeat tracts (1 ± 1%) nor were loops observed following psoralen/UV cross-linking and surface spreading of the DNA. Furthermore, the only TRF2 protein bound was present along the internal 500 bp repeat tract. These results suggest that both the ss and ds portions of the overhang must be telomeric for TRF2 to localize to the ss/ds junction and for t-loops to be assembled.

T-loop formation does not require homology at the 3′-end of the telomere tail

The experiments described above demonstrate that homology between the overhang and the duplex tract is essential for TRF2-mediated t-loop formation. To determine whether homology is required throughout the overhang, three 3′ tails were synthesized, beginning with different numbers of TTAGGG repeats and terminating in non-telomeric sequences: $(TTAGGG)_8(N)_{10}$, $(TTAGGG)_2(N)_{42}$ and $TTAGGG(N)_{48}$ (Table I). The model telomere$_{500bp}$ DNA molecules with these tails formed t-loops at frequencies of 8 ± 5% (n = 100/sample, three experiments), 14 ± 6% and 15 ± 8%, Table I) (n = 100/sample, four experiments), respectively.

The pattern of TRF2 binding to these templates (Table I) suggested a decrease in the amount of TRF2 bound to the end of the telomeric tract (24 ± 17%, 11 ± 4% and 8 ± 3%, respectively) as the number of non-telomeric repeats increased from 10 to 42 to 48, respectively. The DNA with a single 6 nt TTAGGG 3′ overhang (see above) showed the same level of end binding (8%) as the DNA with a single TTAGGG repeat followed by 48 nt of non-telomeric sequence, further pointing to the importance of the sequences at the junction in loading TRF2. The level of end binding for this DNA is also equivalent to the end binding of $N_6(TTAGGG)_8$. Unlike the latter molecule, however, the $TTAGGG(N)_{48}$ DNA forms t-loops with high efficiency. These results would argue further for the importance of both the ss and ds sequences at the very junction in t-loop formation. The amount of TRF2 bound internally along the 500 bp tract was relatively unchanged

(19 ± 15%, 25 ± 11% and 24 ± 3%, respectively; n = 100/sample, three experiments).

Discussion

A looped back structure termed a t-loop has been proposed to sequester chromosome ends from cellular checkpoint proteins and repair enzymes (Griffith *et al*., 1999). In this study, model telomere DNAs and purified TRF2 protein were used to examine the features of the telomere end required for loop formation *in vitro*. We found that at least one ss TTAGGG repeat adjacent to the telomeric duplex is required for efficient t-loop formation. If the ss tail began with TTAGGG repeats but terminated with a non-telomeric sequence at its 3′ end, only a modest reduction in looping was found, suggesting that the terminal 3′ nucleotides need not be complementary as long as they follow a tract of TTAGGG repeats. In contrast, placing non-telomeric sequences on the tail at the ss/ds junction abolished looping even when the remaining tail consisted of TTAGGG repeats. Similarly, loops were not formed with templates containing non-telomeric sequences in the duplex portion of the junction or with blunt-ended telomeric DNA. TRF2 was also unable to form loops when the 3′ overhang was of the C-rich sequence oriented such that it was capable of base pairing with the internal duplex telomeric DNA. There was no significant variation in the ability of TRF2 to bind to the internal portion of these different substrates, and internal binding was not correlated with t-loop formation. Examination of the pattern of TRF2 localization in these experiments revealed that TRF2 most often binds as a large oligomeric form and that there was a strong correlation between the ability of TRF2 to interact with the end of the DNA and the fraction of molecules arranged in t-loops. These results show that TRF2 can behave as a structure-dependent telomere binding protein with a strong tendency to interact near or at the telomeric ss/ds junction at the telomere terminus.

Throughout this work, a maximum of 15–20% of the input DNAs, as scored by EM, were assembled into t-loops by TRF2. There are several possible explanations for why this value did not approach 100%. As shown in Figure 3A–C, the mass of the TRF2 complex bound to the DNA was frequently large and this could have obscured very small loops. Aggregates of the DNA and TRF2 also may have sequestered some of the looped molecules. The instances of two DNA molecules associated end-to-end may represent an intermolecular form of a t-loop structure or a tendency to associate two distinct telomere repeat tracts, a property observed with TRF1 (Griffith *et al*., 1998). The larger aggregates appeared to involve a non-specific association of many DNA molecules by a very large mass of TRF2 protein, and the formation of these aggregates reduced the available level of both telomere ends and TRF2 protein.

A number of the properties of TRF2 are suggestive of a helicase-like action, in particular the formation of a D-loop, which requires separation of the duplex strands. A hallmark of many helicases is the requirement for a single-strand tail of a defined polarity (3′ or 5′, depending on the enzyme) to load onto the duplex. TRF2 was found to have a strong preference for binding near telomeric ds DNA when it contained a 3′ ss tail (with TTAGGG at the ss/ds junction). In addition, as described above, the ability of a 6 nt TTAGGG-3′ tail to generate a t-loop which can be covalently fixed with psoralen and UV argues that the duplex portion of the telomere terminus may have been opened by TRF2 to allow longer segments of the G-rich and possibly C-rich strand to stabilize the D-loop. Homology searches failed to reveal significant amino acid sequence similarity to any helicase. Furthermore, unlike helicases, no requirement for nucleotide hydrolysis or binding was observed in TRF2 end binding or t-loop formation (data not shown). However, since TRF2 may not be released from the D-loop once the duplex has been unwound, unwinding might be driven by a conformational change in the protein, as contrasted with ATP hydrolysis, which would be used to recycle the TRF2. Indeed, RecA will catalyze strand invasion in the absence of ATP hydrolysis, although ATP is required to recycle the protein (Cox, 1994).

An analogy with RecA protein in its catalysis of the search for homology and strand insertion may be instructive in providing parallels for t-loop formation by TRF2. This analogy was further supported by the TRF2-dependent synapsis of two molecules joined together within their telomeric tracts and observed after removal of TRF2 and psoralen/UV cross-linking. RecA protein first assembles as a highly oligomeric complex onto ssDNA; this complex then physically scans dsDNA for sites of homology. Once identified, the first homologous joints formed, termed paranemic, are metastable, being lost upon deproteinization, and they do not involve insertion of the ssDNA into the dsDNA. It is presumed that the paranemic joints are mobile, being able to move along the two DNA molecules until a free homologous end is encountered, upon which strand insertion ensues with the formation of a stable plectonemic joint (reviewed in Griffith and Harris, 1988; Cox, 1994).

In vitro, t-loop formation by TRF2 could follow a similar set of steps. We observed that TRF2 strongly prefers to load as an oligomeric form onto telomeric DNA and to localize at the DNA terminus if it contains at least one 3′-TTAGGG next to duplex telomeric DNA. This localization may occur either by direct end loading or by internal loading followed by migration to the ss/ds junction. Loop formation may be initiated by one of several mechanisms, as modeled in Figure 4. T-loops could form via a single end-bound complex interacting with an internal duplex telomeric segment, or by interacting with a second TRF2 complex bound along the telomeric duplex. It is also possible that a tiny loop is generated within the TRF2 complex, which then grows in size as the loop slips along the dsDNA away from the telomere end. The observation of synapsis of two DNA molecules at their ends (of the order of a half to a quarter of the number of looped species), both in the presence of TRF2 and following cross-linking and deproteinization, argues for this model in which the ss overhang with TRF2 bound at the junction can invade either *in cis* or *in trans*, but more frequently the former due to the proximity of duplex TTAGGG repeats. By analogy with the RecA-driven events, it seems possible that metastable complexes exist in which the telomere end is held back such that it can interact with the internal duplex telomeric repeats but in which no D-loop has formed. Such a complex might be

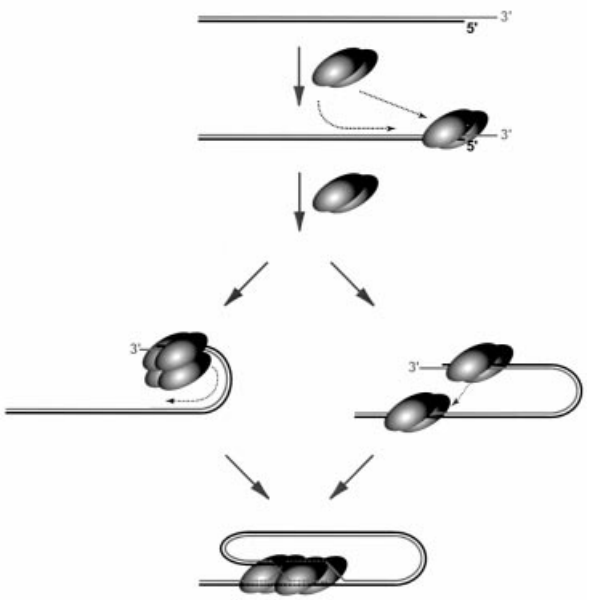

Fig. 4. Model of t-loop formation by TRF2. The results support a model in which the initiating step is the assembly of a TRF2 complex at the ss/ds telomeric junction either by direct binding or by sliding from an internal site. Once formed, loops could be generated by one of several routes, two of which are illustrated here. The junction-bound TRF2 complex may fold backwards to bind an internal site on the telomeric duplex to form a loop (left). Alternatively (right), a second TRF2 complex assembled at an internal site may interact with the end-bound complex to form a loop. A scheme involving a sliding clamp from which a loop grows remains a formal possibility, but is not favored by the observation of two molecules joined *in trans*. While we have shown a sequential assembly of additional TRF2 during loop formation, the masses of protein at each stage remain to be defined more precisely. In both cases, the loop facilitates the invasion of the overhang into the duplex tract to form a t-loop (bottom).

able to move along the DNA, possibly even sliding onto non-telomeric duplex segments before dissociating. Strand insertion, by this model, would then lead to a stable, relatively non-mobile joint. Hence, the looped molecules observed here by EM with TRF2 at the loop junction could be a mix of ones in which the 3′ tail had inserted into the ds telomeric duplex and ones in which it had not.

In vivo, it is likely that t-loop formation involves the participation of other proteins, including TRF1. As modeled in Griffith *et al*. (1999), TRF1 may be involved in the early steps of folding the telomeric DNA back on itself and then may aid in compacting the telomeric chromatin. Furthermore, the Mre11 complex, which is recruited onto telomeres by TRF2 (Zhu *et al*., 2000), has been proposed to play a role in t-loop metabolism. The work described here provides a first step toward understanding telomere remodeling *in vitro* and *in vivo*.

Materials and methods

Oligonucleotides and construction of model telomere DNA molecules

To create a plasmid containing a block of telomere repeats flanked by asymmetric restriction sites, an oligonucleotide containing nine telomeric repeats and *Bbs*I and *Bsm*BI sites was synthesized [5′-CCCGGA-TCCGTCTCA(CCCTAA)$_9$GTCTTCAAGCTTCCC-3′] and annealed to a complementary oligonucleotide (5′-GGGAAGCTTGAAGACTT-3′) by mixing equimolar quantities, heating and slowly cooling to room temperature over a 12 h period. The annealed oligonucleotides were converted to an 84 bp fragment by a Klenow fill-in reaction. This fragment was then cloned into the *Bam*HI and *Hin*dIII sites of pBluescript II SK+ (Stratagene Inc.) according to standard protocols. The reaction mixture was transformed into *Escherichia coli* Sure2 cells (Stratagene). The resulting plasmid, pRST15, was identified by the blue/white color test and confirmed by direct sequence analysis (Iowa State University Nucleic Acid Facility).

To generate the model telomere$_{500bp}$ DNA, the telomeric fragment was isolated from the pRST15 clone by *Bbs*I–*Bsm*BI digestion, followed by agarose gel isolation (Qiagen Inc.). The insert was ligated into *Bsm*BI-digested pRST15 and cloned as above. The cycle was repeated to obtain a plasmid with a tract of a fixed size, ~500 bp (pRST5). The exact number of repeats is not known, but sequence analysis placed it between 85 and 98 repeats.

To generate the model telomere$_{\pm 2\ kb}$ DNA, pRST5 was digested with *Bsm*BI, placing the telomere repeats at the end of the linearized plasmid. Replication was performed using a 14 telomeric repeat-containing oligonucleotide [5′-(CCCTAA)$_{14}$-3′] to unidirectionally extend the telomere to an average length of 2 kb, as determined by EM and gel analysis. The oligonucleotide and the plasmid were combined at a 1:2 mass ratio in a 100 µl PCR reaction containing 50 mM Tris–HCl pH 9.1, 16 mM NH_4SO_4, 3.5 mM $MgCl_2$, 150 µg/ml bovine serum albumin (BSA), 250 µM dNTPs, Klentaq (5 U) (Clontech. Inc) and *Pfu* polymerase (0.03 U) (Stratagene Inc.). Conditions for elongation were: 96°C for 1 min, 64°C for 1 min, 72°C for 1.5 min, three cycles; 96°C for 1 min, 64°C for 1 min, 72°C for 2.5 min, three cycles; 96°C for 1 min, 64°C for 1 min, 72°C for 3.5 min, two cycles; 72°C for 7 min. The resulting DNA was purified and treated with mung bean nuclease (9 U) at 37°C for 30 min to remove any ss regions. A 3′ overhang of 75–200 nt, as measured by EM (see Griffith *et al*., 1999), was generated by digesting with T7 gene 6 exonuclease (2 U) (USB Amersham Inc.) at 16°C for 8 min in a 10 µl reaction containing 40 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$ and 50 mM NaCl.

The interrupted model telomere template was generated from pRST5 DNA by digestion with *Ava*II and *Sca*I to place the telomeric repeat tract 1124 bp from the *Sca*I site and 1465 bp from the *Ava*II site. A 3′ overhang GTC(TTAGGG)$_{12}$ was ligated to the *Ava*II–*Sca*I fragment as described below. All restriction endonucleases and enzymes were from NEB Inc., unless noted otherwise.

Ligation of oligonucleotide tails to pRST5

Oligonucleotides of varying sequence and orientations, each containing a 5′ phosphate and most containing two internal biotin moieties, were purchased from MWG Biotech Inc. The locations of the biotin moieties within each tail are given in terms of the distance of the labeled nucleotide from the 3′-end of the overhang and are listed in parentheses following each labeled oligonucleotide. The sequences were 5′-AGGG(TTAGGG)$_9$ (16, 40), AGGG(TTGGGG)$_9$ (16, 40), AGGG(CCCTAA)$_9$ (14, 38), AGGG(TTAGGG)$_8$CTCGATACGA (20, 44), AGGGTTAGGGTG-AGCTACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCG-CAAA, AGGG(TTAGGG)$_2$TGAGCTACAGCACCAGATTCAGCAA-TTAAGCTCTAAGCCATC, AGGGGTGCGAG(TTAGGG)$_8$, AGG-GGGATGTATGGTGGAGGTGTGAGATGTGTGATGGTGAGGTG-GTATGGTGAGTGT (14, 42), 5′-AGGGTTAGGG, 5′-AGGG-(TTAGGG)$_2$, 5′-AGGG(TTAGGG)$_4$ and 5′-AGGG(TTAG-GG)$_{14}$. The ligation reactions contained 8 µg of linearized plasmid DNA and were performed with a 5-fold molar excess of oligonucleotide at 16°C overnight in 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml BSA and T4 ligase (200 U) (NEB). The free ss oligonucleotides were removed using BND (benzylated naphthoylated DEAE) cellulose (Sigma Inc.). When the model DNA was incubated with streptavidin for 10 min at room temperature, an average of 82% (± 2%) of the DNA molecules showed binding at one end, as determined by EM, confirming addition of the ss tail.

Preparation of DNA–protein complexes and electron microscopy

EM was used to optimize the TRF2 looping reactions. Varying salt, pH, temperature and time of incubation, optimal conditions were found to involve incubation of the DNA at 0.5 µg/ml with TRF2 at room temperature for 20 min in a buffer containing 20 mM HEPES pH 8.75 with 0.1 mM EDTA (HE buffer) and a stoichiometry of three dimers of TRF2 per duplex TTAGGG repeat. Inclusion of dATP, dGTP, rATP or rGTP (1 mM each) with either Mn^{2+} or Mg^{2+} (2 mM each) did not significantly stimulate or inhibit the level of loop formation or TRF2

binding relative to that seen in the HE buffer alone (data not shown). Both TRF1 (Griffith *et al*., 1998) and TRF2 contained a histidine tag and were purified by Ni^{+} chromatography of baculovirus-produced protein generated in insect cells, and preparations generated in both laboratories were used in these studies.

Following incubation, the samples were mixed with a buffer containing spermidine, adsorbed to glow-charged thin carbon foils, dehydrated through a series of water–ethanol washes, and rotary shadow cast with tungsten as described previously (Griffith and Christiansen, 1978). AMT/UV cross-linking was performed as described previously (Griffith *et al*., 1999). Samples were examined in Philips EM400 or CM12 instruments.

The droplet variation of the Kleinschmidt method (Kleinschmidt and Zahn, 1959) was used for surface spreading DNA as described (Griffith *et al*., 1999). Micrographs were scanned from negatives using a Nikon 4500AF multiformat film scanner. The contrast was optimized and panels were arranged using Adobe Photoshop. Lengths of the model telomere$_{\pm 2kb}$ DNA molecules were measured using a Summagraphics digitizer with software developed by J.D.Griffiths. Lengths of the model telomere$_{500bp}$ DNA molecules were measured using Gatan Digital Micrograph 3.3.

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