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Transcription of telomeric DNA leads to high levels of homologous recombination and t-loops

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ABSTRACT

The formation of DNA loops at chromosome ends (t-loops) and the transcription of telomeres producing G-rich RNA (TERRA) represent two central features of telomeres. To explore a possible link between them we employed artificial human telomeres containing long arrays of TTAGGG repeats flanked by the T7 or T3 promoters. Transcription of these DNAs generates a high frequency of t-loops within individual molecules and homologous recombination events between different DNAs at their telomeric sequences. T-loop formation does not require a single strand overhang, arguing that both terminal strands insert into the preceding duplex. The loops are very stable and some RNase H resistant TERRA remains at the t-loop, likely adding to their stability. Transcription of DNAs containing TTAGTG or TGAGTG repeats showed greatly reduced loop formation. While in the cell multiple pathways may lead to t-loop formation, the pathway revealed here does not depend on the shelterins but rather on the unique character of telomeric DNA when it is opened for transcription. Hence, telomeric sequences may have evolved to facilitate their ability to loop back on themselves.

INTRODUCTION

The ends of eukaryotic chromosomes are capped by DNA–protein complexes termed telomeres, which in most species consist of a repeating DNA element bound by general chromatin proteins and telomere-specific factors. Telomeres may be as short as a few hundred base pairs (bp) in yeasts and as long as 150 kb in plants. In humans, telomeres approach 15 kb at birth and diminish as we age. The telomere specific proteins in higher eukaryotes are present in one or more multi-protein complexes termed shelterins, as the DNA at the end of the telomere must be sheltered from erroneous recognition as a double strand break (1). This function is believed to be accomplished by the combination of shelterin binding and an architectural solution in which the DNA terminus folds back to generate a duplex loop (t-loop) that hides the DNA end (2,3).

T-loops have been isolated and visualized by electron microscopy (EM) from species ranging from yeast to humans as reviewed in (2,4). The t-loop junction can occur anywhere along the length of the telomere, generating a spectrum of circle sizes; in peas, t-loops as large as 120 kb with an 80 kb circular portion were observed (5). Recently, using a fluorescent PNA probe to telomeric sequences, STORM imaged fields of t-loops from mouse cells were observed (4) and the dimensions of the t-loop molecules were close to those observed by EM. The percentage of looped species relative to linear telomeric restriction fragments has ranged from 15 to 30% in the EM studies and up to 40% in the STORM analysis.

Our earlier suggestion that telomeres might loop back on themselves (2,3) was based on the fact that most eukaryotic telomeres end with a 3′ single stranded (ss) tail on the G-rich strand which has the same sequence as the preceding duplex (ds) telomeric DNA (6). Based on our understanding of homologous recombination (HR) reactions, the 3′ ss tail would be expected to invade the preceding duplex segment to generate a lasso structure with a D-loop at the junction. This represents a simple intramolecular HR product. Using a model telomere template containing 1–2 kb of TTAGGG repeats and terminating with a 3′ ss tail on the G rich strand, we observed that purified TRF2 protein would generate t-loops in 15–30% of the DNA, but that TRF1 would not (3). Loop formation required the presence of a homologous 3′ G rich ss tail (7), and the junctions were stained with a single strand binding protein (3) pointing to a D-loop at the junction; however, it remained possible that both strands could be inserted. The recent STORM studies showed that using an inducible system, elimination of functional TRF2 in cells resulted in a several fold reduction in the number of t-loop molecules, while elimination of other shelterins, including TRF1 and Pot1, did not (4). Because TRF2 is part of a multiprotein complex, the other shelterin components may work with TRF2 to either actively form t-loops or perhaps stabilize them once formed by a variety of mechanisms. Indeed, in the cell there may be multiple pathways leading to t-loop formation and stabilization and one potential pathway might involve transcription.

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Telomeres from yeast to humans are transcribed by RNA polymerase II from sub-telomeric promoters. The C-rich telomeric strand is predominantly transcribed, generating a G-rich RNA termed TERRA as first described by the Lingner and Blasco groups (8,9). TERRA transcripts vary in length but can be as long as 9000 nt in humans; however, it is possible that much of the TERRA is relatively small (8–10). TERRA is now accepted as a key structural element of telomeres, likely providing a structural scaffold upon which proteins involved in telomere maintenance bind. TRF1 and TRF2 (11), hnRNPA1 (12) and replication factors such as Orc1 (11) bind TERRA. Cell cycle studies show that TERRA peaks in G1, declines through S phase and then rises in G2 (10).

Biological studies have shown that TERRA levels are increased in human cancer cells (13) and in cells employing the ALT pathway (Alternative Lengthening of Telomeres) (14–16). TERRA may be involved in telomere length signaling as depletion of TRF2 and shortening of telomeres lead to elevated levels of TERRA (17,18). Indeed, Porro *et al*. (2014) showed that reducing TRF2 experimentally in cells having long telomeres results in an increase in TERRA, suggesting that TERRA helps remodel telomeres after loss of TRF2 (18).

TERRA transcribed from mammalian telomeres contains repeating runs of 3 guanine bases that can form G quartets. We showed that 576 nt (UUAGGG)n TERRA transcripts are arranged into chains of 24 nt particles linked by a 3 nt bridge (19). Of particular relevance here is the finding that transcription of telomeres can result in RNA–DNA hybrids termed R loops in which the G-rich strand is displaced and TERRA base pairs to the C-rich template DNA strand, as reviewed in (20). Telomeric R loops have been detected at the telomeres of yeast, human and human cancer cells including ALT cells (20). In ALT cells, a direct correlation has been observed between enhanced HR, elevated TERRA levels and repression of RNase H1 (14). R-loops may promote HR by opening the helix and providing an entry site for another homologous DNA strand. Indeed, upon expression of RNase H1 in ALT cells, the level of TERRA diminished and the telomeres were no longer maintained via HR (14). Thus, transcription of a telomere could directly influence its architecture by generating R loops that facilitate intramolecular looping as well as telomere–telomere associations.

To begin to probe these possibilities in an *in vitro* system we employed T7 RNA polymerase and two sets of DNA templates containing the T7 promoter. One contains a 576 bp tract of TTAGGG repeats at the end of a 3.5 kb plasmid and a second consists of very long ds telomeric DNA approaching the size of human telomeres. Transcription leads to a high frequency of HR products consisting of highly stable t-loops and molecules that have undergone intermolecular recombination at their telomeric sequences. This work suggests that t-loop formation is driven by the unusual nature of telomeric DNA when it is opened for transcription and that shelterin proteins may have evolved to protect the loops once formed by a variety of mechanisms.

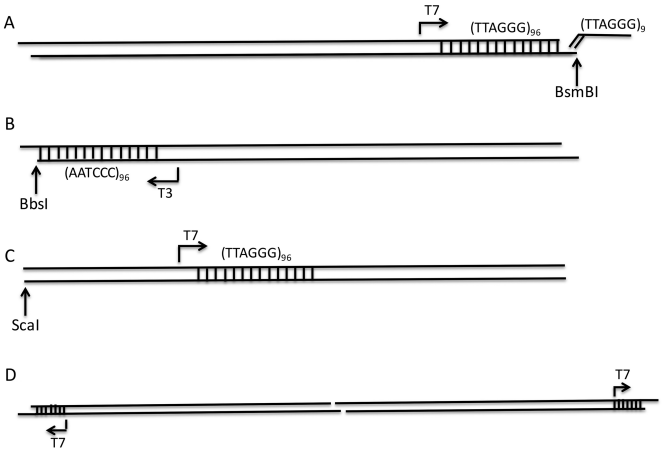

Figure 1. DNA templates. The pRST5 plasmid (7) contains a 576 bp track of $(TTAGGG)_n$ repeats in a pGEM background. (**A**) Cleavage with BsmBI generates a linear DNA with the repeats at one end and the T7 RNA polymerase promoter at the beginning of the repeats oriented as shown. The 4 nt 5′ overhang allows ligation of ss tails. (**B**) Cleavage with BbsI results in the opposite orientation and the T3 RNA polymerase promoter oriented as shown. (**C**) Cleavage with ScaI places the repeats in a central location. (**D**) Ligation of the linear DNA molecules shown in A generates a miniature chromosome with the telomeric sequences at both ends.

MATERIALS AND METHODS

Plasmid DNAs

Construction of pRST5 (Figure 1) containing 576 bp of TTAGGG repeats and T7/T3 promoters was previously described (7,19). In some experiments the linear DNA contained 54 nt or 94 nt 3′ TTAGGG overhangs (oligonucleotides synthesized by MWG Operon Inc.).

Proteins

For some experiments transcription was carried out using the T7 and T3 Maxiscript kits from Ambion Inc. In addition, T7 RNA polymerase was purified in this laboratory from a plasmid containing a hexahistidine-tagged form of T7 RNA polymerase expressed in *E. coli* (gift of laboratory of Thomas Cech) using their purification protocol. TRF1 and TRF2 proteins were purified and characterized in this laboratory as N-terminally hexahistidine tagged forms, previously described (21). The *Escherichia coli* expression vectors generated (deposited in Addgene.org, Cambridge, MA, plasmid #53209 TRF1 and plasmid #50488 for TRF2) were based on vectors generously provided by the laboratory of E. Gilson (University of Nice, Nice, France). Both proteins were purified by multiple HisTrap HP, HiTrap Heparin HP and HiTrap QFF columns using an AKTA purifier FPLC (GE Healthcare Lifesciences) and were free of any detectible nucleases.

Generation of long telomeric dsDNA

dsDNA containing the T7 RNA polymerase promoter followed by up to 15 kb of pure TTAGGG repeats or mutant sequences were constructed as follows. A 96 nt oligonucleotide consisting of 5′CCCTAA3′ repeats (wild type), 5′CACTCA3′ (telomeric sequence mutant) or

5′CACTAA3′ (quadruplex mutant) was circularized using CircLigase (Epicentre, Illumina) under conditions described by the vendor. A 115 nt oligonucleotide consisting of the T7 promoter (5′TAATACGACTCACTATAGG3′) followed by 16 5′TTAGGG3′ repeats (wild type), 5′TGAGTG3′ repeats (telomeric sequence mutant) or 5′TTAGTG3′ repeats (quadruplex mutant) (Eurofins) was annealed to the circle to generate a dsDNA circle with a displaced tail containing the T7 promoter. Incubation for 16 h at 30°C with ϕ29 DNA polymerase (New England Biolabs) in a buffer containing 10 mM dNTPs yielded G-rich telomeric ssDNA up to at least 15 000 nt long as measured on agarose gels. The newly synthesized DNA was deproteinized with 20 μg Proteinase K (Invitrogen), 0.02M EDTA and 0.4% SDS in a 50 μl volume for 1 h at 55°C and purified by ethanol precipitation for further processing. A complementary 90 nt oligonucleotide consisting of 15 5′CCCTAA3′ (wild type), 5′CACTCA3′ (telomeric sequence mutant) or 5′CACTAA3′ (quadruplex mutant) repeats (Eurofins) was then annealed following heating to 100°C to release the small circle and the sample was then incubated with DNA polymerase I (New England Biolabs) for 2 h following the manufacturer's protocol. This newly synthesized dsDNA was deproteinized with 20 μg Proteinase K (Invitrogen), 0.02M EDTA and 0.4% SDS in a 50 μl volume for 1 h at 55°C and purified by DNA Clean & Concentrator^{TM}-25 kit (Zymo research). The size of the resulting telomeric dsDNA was analyzed by 0.8% agarose gel electrophoresis and measured by EM.

Transcription of the telomere templates

Using the commercial T7 or T3 Maxiscript kits (Ambion Inc.) transcription was carried out at 37°C for 1 to 60 min in the buffers supplied by the vendor. Transcription by T7 RNA polymerase purified in this laboratory was carried out using a buffer containing: 40 mM Tris-Cl, 10 mM $MgCl_2$, 4 mM spermidine, 10 mM DTT, 10 mM NaCl for 5 to 60 min. Typical transcription reactions contained 100 ng of DNA in a 30 μl volume. Following incubation of the DNA at 37°C, the DNA was treated with 70 μg/ml trioxsalen (Sigma) and long wave UV (∼360 nm, 1 cm from sample) to crosslink the DNA, followed by treatment with 660 μg/ml RNase A (Sigma) or in some experiments 10 units of RNase H (New England Biolabs) or 100 units of RNase T1 (Thermo Scientific) followed by addition of Proteinase K (500 μg/ml), SDS (to 0.5%) and EDTA (5 mM) and incubation for 60 min at 37 or 55°C. The DNA was then passed over 2 ml columns of 2% agarose beads (Agarose Bead Technologies) previously equilibrated in 10 mM Tris pH 7.5, 0.1 mM EDTA. The DNA in the excluded volume was examined by EM. In some transcription experiments, the pRST5 DNA was treated with 10U S1 nuclease (Thermo Scientific) for 30 min at room temperature, followed by enzyme inactivation and DNA cleaning with DNA clean and concentrator kits (Zymo Research) prior to transcription. Transcription efficiencies of different long template DNAs were checked after transcription by treating them with TURBO DNAse (Ambion) as per manufacturer's protocol and running the samples on a 1% agarose gel. The electrophoresis was run at 140 V in a 10 cm tray and the gel stained with SYBR Gold (Thermo Scientific) and examined in a UV transilluminator (BioRad.).

Iron particle staining of TERRA

Transcription reactions with pRST5 DNA were carried out for 30 min at 37°C using ribonucleotides at 10 mM concentration including a mixture of biotin-16-UTP (Roche Diagnostics GmbH) and unmodified UTP at a molar ratio of 1:4. The samples were then treated with RNase A and H for 1 h at 37°C followed by deproteinization with Proteinase K and SDS at 37°C. The samples were filtered through agarose beads and the peak fractions pooled. Paramagnetic iron particles (μMACS streptavidin microbeads, Miltenyi Inc.) (3 μl) were added to a 50 μl aliquot of the purified t-loop molecules and allowed to incubate overnight at 4°C. The samples were then filtered through agarose beads to remove the unbound beads and prepared for EM.

Binding and transcription with TRF1 and TRF2

Preincubation of pRST5 with TRF1 or TRF2 was done in a buffer of 20 mM Hepes, pH 8.0, 150 mM KCL, 1 mM DTT and 0.1 mM EDTA. For TRF1, 480 ng of protein were added to 100 ng of DNA, and for TRF2, 72, 144 or 288 ng of protein were incubated with 100 ng of DNA for 20 min at room temperature. Salts were then increased to achieve 40 mM Tris pH 7.5, 10 mM $MgCl_2$, 4 mM spermidine, 10 mM DTT, 10 mM NaCl. Ribotriphosphates followed by RNA polymerase T7 were added.

Gel electrophoresis

Transcription reactions were carried out with 100 ng DNA substrate in a volume of 20 μl, processed and deproteinized as carried out for EM and loaded on a 0.8% agarose gel in TAE buffer. Electrophoresis was run at 140 V in a 10 cm tray and the gel stained with SYBR Gold (Thermo Scientific) and examined in a UV transilluminator (BioRad.).

Electron microscopy

DNA samples to be examined by spreading in a monolayer of denatured cytochrome C at an air-water interface were prepared as described (22) including rotary shadow casting in a high vacuum with platinum–palladium. DNA and DNA–RNA complexes to be examined directly were adsorbed to thin carbon foils in the presence of spermidine, washed with a water-ethanol series, air dried and rotary shadow cast with tungsten at 10^{-7} torr (23). Images were captured using a Gatan Orius real time CCD camera (Pleasanton, CA, USA) attached to an FEI Tecnai T12 TEM/STEM instrument (Hillsboro, OR, USA) operated at 40 kV.

RESULTS

Transcription of telomeric DNA produces high levels of t-loops and HR products

The pRST5 plasmid contains a 576 bp block of TTAGGG repeats in a pGEM background (Figure 1). The repeats are

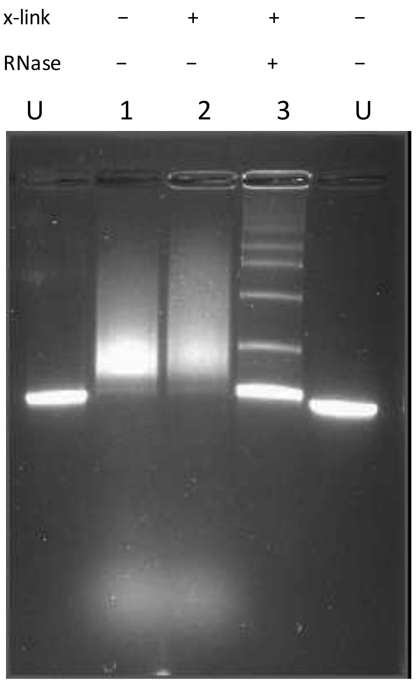

Figure 2. Agarose gel electrophoretic analysis of transcription mediated events. Agarose gel electrophoresis of linear pRST5 (BsmBI cut, no 3′ overhang) DNA in transcription buffer alone (U). If the DNA was transcribed for 30 min (Materials and Methods) and then deproteinized but not treated with RNase A, the DNA with RNA bound was present as a smear whether or not it was crosslinked with psoralen and UV (1, 2). Following transcription, deproteinization and treatment with RNase A (3) the DNA was present as a ladder of bands with the monomer band shifted upward.

flanked on one side by a T7 promoter and on the other by a T3 promoter. Cleavage by BsmBI places the TTAGGG repeats at one end of the DNA leaving a 4 nt 5′ overhang on the C rich strand allowing ligation of oligonucleotides to generate 3′ TTAGGG ss overhangs on the G-rich strand. Transcription by T7 RNA polymerase generates G-rich TERRA (19). Cleavage with BbsI places the repeats at the other end with the T3 promoter oriented into the repeats and transcription by T3 RNA polymerase generates C-rich transcripts. Ligation of the BsmBI cut DNA will generate dimeric molecules with the T7 promoter and telomeric tracts at both ends. In all T7 RNA polymerase transcriptions below (unless otherwise noted) the pRST5 template was cleaved with BsmBI.

Linear pRST5 containing a 54 nt $(TTAGGG)_9$ 3′ overhang was transcribed with T7 RNA polymerase at 37°C for 30 min (using the enzyme purified in this laboratory or the Ambion Megascript kit with similar results) followed by photo crosslinking with psoralen and UV. The samples were then deproteinized with Proteinase K and SDS. Agarose gel electrophoresis (Figure 2, lanes 1,2) showed that irrespective of crosslinking, the linear DNA appeared as a smear of slower migrating material due presumably to RNA remaining bound to the DNA. When the samples were treated with RNase A (30 min, 37°C) following transcription, and then deproteinized, a distinct ladder of bands was observed (Figure 2, lane 3). The most rapidly migrating band was at a position slightly retarded from the non-transcribed DNA and this was followed by sharp multimer bands, as well as material near or in the well of the gel.

To directly image DNA in these experiments, following deproteinization the samples were filtered through agarose beads to separate the nucleic acids for EM examination (Materials and Methods). The DNA was spread on a film of denatured cytochrome C protein at an air–liquid interface ('surface spreading', Materials and Methods). Examination of samples that had not undergone RNase A treatment (as in Figure 2, lane 2), revealed fields in which, in one experiment, 92% (n = 212) of the linear DNA contained an RNA 'bush' at one end (Figure 3A). When the RNase A treatment was included (as in Figure 2, lane 3), the RNA was no longer visible and up to one half of the linear DNAs showed a small loop at one end (Figure 3B) (Table 1A). In addition, multimers involving 2, 3 or more DNAs joined at one end (Figure 3C) were present, as well as clusters in which a large number of DNAs were fused at one end leaving the other end spread out from a dense central core (Figure 3D). We termed these 'DNA bouquets'. From 25 to 50% of the DNA was present in multimers and bouquets, which were not seen in the absence of transcription. The EM results thus correlated perfectly with the results from agarose gel electrophoresis and the slight retardation of the monomer band is presumably due to the end being looped.

To demonstrate that the small loops and sites where the multimers were fused were at the telomeric sequences, samples were prepared as above (Figure 3B–D) and then treated with NaeI. This generates a 935 bp fragment containing 359 bp of plasmid sequences followed by the TTAGGG repeats. EM revealed ∼1 kb fragments containing the loops (Figure 3E) and bouquets with the DNA arms reduced to ∼1 kb (Figure 3F). Mapping the location of the loops along the pRST5 DNA showed that the loops were localized exclusively to the telomeric sequences with a distribution of loop sizes between 200 and 550 bp and a peak near 400 bp (Supplementary Figure S1). Using a miniature chromosome containing the telomeric tracts at both ends (Figure 1D), transcription generated frequent molecules with small loops at both ends (Figure 3G).

When pRST5 DNA with the 54 nt 3′ overhang was incubated for 30 min in the transcription buffer without RNA polymerase and then prepared for EM, only 4% (n = 338) of the linear DNAs had a loop at one end. If RNA polymerase was included but the incubations lacked rNTPs, or contained ATP and UTP but not GTP, the percentage of DNAs with a loop at one end was 1% (n = 100) and 1% (n = 101), respectively, and multimers or bouquets were not observed. Incubation of pRST5 DNA (100 ng) containing the 54 nt overhang for 30 min at 37°C with 100 ng of purified TERRA under transcription conditions but without RNA polymerase did not generate t-loops (2% looped, n = 200) (Table 1A).

The transcription reactions generated a consistently high frequency of t-loops. Over the course of the experiments described in this report, in 15 experiments employing pRST5 with a 54 nt 3′ ss overhang scoring monomer DNAs (not in multimers), the percentage with a loop at one end ranged from 35% to 54% (1954 total molecules scored) (Table 1A). When circular pRST5 was cleaved with ScaI to place the TTAGGG repeat block in the center (Figure 1C), no exam-

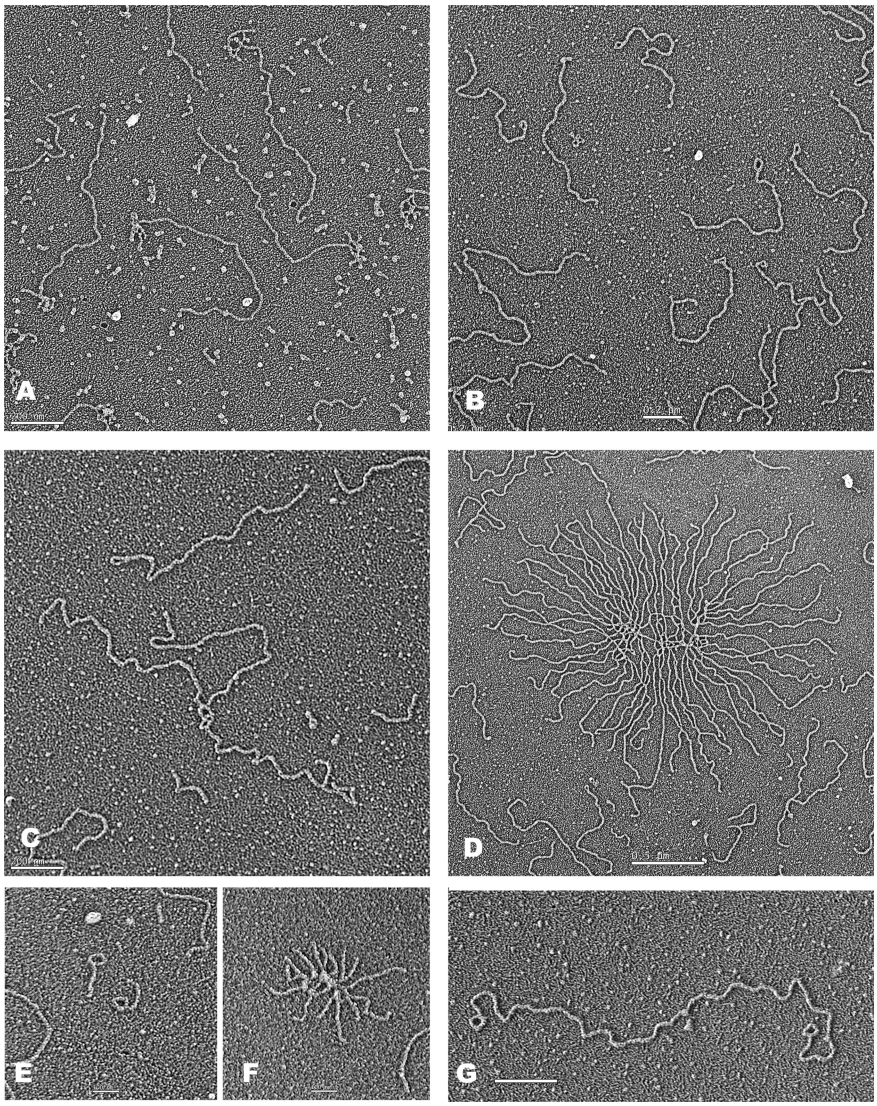

Figure 3. EM visualization of transcription induced loops and HR products. (**A**) If the DNA templates were transcribed but not treated with RNAse A prior to preparation for EM, over 70% of the DNA had a bush of RNA attached at one end (see text for details). (**B**–**G**) With RNase A treatment, fields of linear molecules were present many of which contained a (**B**) tiny loop at one end and in the same fields examples of (**C**) several DNAs joined at one end were present, along with DNA bouquets containing (D) many molecules fused at one end. (**E**) When the samples above (B–G) were cleaved with NaeI that cuts 935 bp in from the telomeric end ∼1 kb fragments with loops (E) as well as bouquets with ∼1 kb arms (F) were observed. (**G**) Transcription of the minichromosome template shown in Figure 1D and processing as in B yielded DNAs with loops at both ends. Samples were prepared by surface spreading with cytochrome C (Materials and Methods) followed by rotary metal shadow casting. Bars indicating magnification are shown in each panel.

ples were seen following transcription in which one or both ends were fused back to the middle of the DNA. However, when this DNA was mixed with an equal amount of linear DNA with the telomeric repeats at the end, and the mixture transcribed, bimolecular HR products were observed in which the end of the DNA with the terminal repeats invaded the middle of the DNA with the central repeats (Supplementary Figure S2A). This argues that the multimers and bouquets involve HR strand invasions at the telomeric sequences. While most of the incubations were carried out at 37°C for 15 or 30 min, a significant number of looped DNAs were seen as early as 1 and 2 min (14% and 23%, respectively), with an increase over the remaining 60 min. Agarose gel electrophoresis verified that the dimer, trimer and multimer bands were only seen following transcription, and further, that these bands began to appear as early as 2 min after initiation of the reactions (Figure 4, lanes 1–3).

Transcription-mediated looping does not require a 3′ ss overhang

To ask whether t-loop formation in these reactions requires 3′ G-rich ss tails, pRST5 containing a 4 nt 5′ extension at the telomeric end (Figure 1A) was transcribed for 30 min with T7 RNA polymerase in two separate experiments and processed for EM. The fraction of looped DNAs was 55% (n = 165) and 45% (n = 150), and the fraction of DNA present in multimers was similar to that seen above. Agarose gel electrophoresis of the DNA with a 4 nt 5′ extension showed the same pattern of shifted monomer and multimer bands as

Table 1. Summary of EM scoring of over 6500 single molecules from the transcription experiments. Multiple entries in the rows represent replicate experiments under the same conditions but done on different days. Corresponding number of molecules scored for each is listed in order in the left column. X-link refers to psoralen and UV photo crosslinking. See text for specific details

(A)

DNA substrate/conditions	T7 RNA pol	T3 RNA pol	All rNTPs added	Multimers/bouquets	Loops seen%	Molecules counted (n=)
pRST5 + 54 nt overhang (oh)	–	–	+	no	4	338
pRST5 + 54 nt oh	+	–	–	No	1, 1	100, 101
pRST5 + 54 nt oh+ TERRA	–	–	+	no	2	200
pRST5 + 54 nt oh	+	–	+	yes	41, 48, 42 44, 40, 41, 46, 47, 40, 41, 47, 54, 52, 44, 35	125, 105, 113, 122, 140, 160, 102, 187, 168, 98, 75, 123, 245, 68, 123
pRST5(BsmBI) (4 nt 5′ ext)	+	–	+	yes	55, 45	165, 150
pRST5(BsmBI)+S1 nuclease	+	–	+	yes	38	165
pRST5(BbsI)	–	+	+	yes	35, 46, 47	153, 168, 99
pRST5 + 94 nt oh, no X-link	+	–	+	yes	27	200
pRST5 + 4 nt 5′ ext, no X-link	+	–	+	yes	28	200

(B)

DNA substrate/conditions	T7 RNA pol	All rNTPs added	Branched	Loops seen %	Molecules counted (n=)
G-rich DNA 5′(TTAGGG)n3′	+	+	yes	28.5, 27, 28	223, 211, 205
G-rich DNA	–	+	yes	4, 4.5, 4	169, 111, 219
G-rich DNA	+	–	no	6.5, 5, 4.5	197, 194, 239
G-rich DNA +S1 nuclease	+	+	yes	27,26,26	243,208,223
Quadruplex mutant DNA 5′(TTAGTG)$_n$3′	+	+	yes	12, 11, 9.5	143, 119, 123
Sequence mutant DNA 5′(TGAGTG)n3′	+	+	yes	16, 11, 14	131, 111, 126

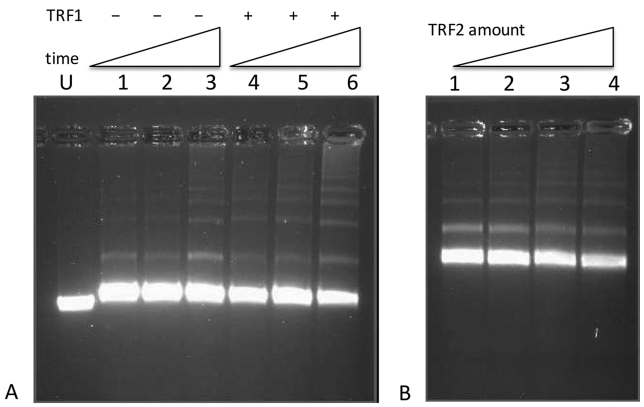

Figure 4. Agarose gel electrophoretic analysis of DNA transcribed in the presence of TRF1 or TRF2. (**A**) Agarose gel electrophoresis of linear pRST5 (BsmBI cut no 3′ overhang) DNA in transcription buffer alone (U). Lanes 1–6: the same DNA transcribed with T7 polymerase for for 2 min (1, 4), 5 min (2, 5) and 20 min (3, 6), then treated with RNase A, and deproteinized. In lanes 4–6 the DNA was preincubated for 20 min with TRF1 protein (text, Materials and Methods) prior to transcription. (**B**) pRST5 DNA (BsmBI cut, 54 nt sss 3′ overhang) was preincubated with 0, 2, 4 or 8 TRF2 monomers per terminal ss/ds junction, respectively, (lanes 1–4) prior to transcription for 30 min with T7 RNA polymerase, RNase treatment and deproteinization.

the DNA with a 54 nt 3′ tail (data not shown). Fully blunt ended DNAs were also efficient templates for loop formation as transcription of S1 treated DNA yielded 38% (n = 165) of the linear DNA having terminal loops (n = 165) (Table 1A).

Cleavage of pRST5 with BbsI produces a linear DNA with a 4 nt 5′ extension and with the telomeric repeats at the other end as contrasted to BsmBI cleavage (Figure 1B). This DNA contains a T3 RNA polymerase promoter at the beginning of the repeats oriented to produce a C-rich telomere transcript. Upon transcription with T3 RNA polymerase (Materials and Methods) in three separate experiments the percentage of monomer DNAs with a loop at one end was 35%, 46% and 47% (n = 420) (Table 1A). Multimers were present at a similar frequency as in the experiments above. These results show that transcription mediated t-loop formation can occur using two different RNA polymerases, and provides another example in which a 3′ ss overhang was not required.

Transcription mediated t-loops are highly stable

Psoralen photo crosslinking DNA has been a long standing method for fixing structures such as replication forks (24,25) and t-loops against loss by branch migration. To examine the stability of t-loops that had not been photo crosslinked, linear pRST5 (with a 54 nt 3′ ss overhang) was transcribed with T7 RNA polymerase for 60 min. One aliquot was UV photo crosslinked while the second aliquot was not. Both were treated with RNase A, deproteinized and chromatographed over agarose beads and allowed to remain at 4°C for 60 h before EM examination. For the sample crosslinked at t = 1 h, 61% (n = 109) of the DNA not in multimers were in t-loops, while the sample crosslinked at t = 60 h showed 48% looped molecules (n = 130). This corresponds to only a 20% loss of t-loops over 60 h at 4°C following an initial treatment with RNase A. In an experiment comparing T7 and T3 transcriptions, the same DNA was crosslinked at t = 0.5 h or at t = 20 h following a 30 min transcription, treatment with RNase A and RNase H (specific for RNA–DNA hybrids), deproteinization and agarose bead chromatography. EM analysis showed that the fraction of looped molecules declined from 50% (t = 0.5 h) to 26% (t = 20 h) for the T7 transcribed samples and from 35% (t = 0.5 h) to 16% (t = 20 h) for the T3 transcriptions, a 48% and 54% drop respectively over 20 h at 4°C following a more stringent RNase treatment. To examine the stability of the

loops when the RNA remained present, pRST5 with a 54 nt 3′ ss overhang was transcribed with T7 RNA polymerase for 30 min followed by deproteinization and agarose chromatography, but not treated with RNase A or H. The sample was then left at 4°C for 72 h followed by photo crosslinking and RNase A treatment. A high percentage of t-loops was observed (57% of the monomer DNA) which was as high as seen in experiments when crosslinking was done at the end of the transcription reactions. In other studies (data not shown) t-loops were equally stable with 4 nt 5′ extensions as with the 54 nt 3′ extension.

It was of interest to examine the thermal stability of the t-loop molecules. Linear pRST5 (4 nt 5′ extension) was transcribed for 30 min and the DNA processed for EM but without photo crosslinking. Aliquots were then heated at 21°C, 37°C, 42°C, 55°C and 65°C for 20 min followed by preparation at room temperature for EM by direct adsorption. As the temperature increased, the percentage of monomers in t-loops declined: 46% (n = 102) at 21°C, 24% (n = 75) at 37°C, 25% (n = 141) 42°C, 16% (n = 128) at 55°C and 14% (n = 129) at 65°C, revealing a significant thermal stability. From these experiments we conclude that transcription-mediated t-loops are highly stable, further arguing that both strands are inserted back into the preceding telomeric duplex. This high stability will open the door for future experiments using non-crosslinked t-loop preparations to probe their resolution by junction resolving enzymes.

TRF1 and TRF2 do not impede looping in these reactions

It was of interest to carry out initial investigations of how preincubation of pRST5 with TRF1 or TRF2 might affect its subsequent transcription. Clearly a detailed study for the future will require a eukaryotic RNA polymerase II transcription system and a chromatinized DNA template. The TRF2 protein used was previously characterized in an *in vitro* strand assimilation assay (21) and the amount used (2, 4 and 8 protein monomers per telomeric ss/ds junction) was based on previous studies (26) and on an EM determination of the maximal amount of TRF2 that could be added without generating large DNA aggregates. The DNA containing a 54 nt 3′ overhang was preincubated at room temperature for 20 min with TRF2 in binding buffer and then transcribed with T7 RNA polymerase following addition of salts optimal for transcription (Materials and Methods). Parallel experiments were carried out using TRF1. Incubation conditions were taken from our study of TRF1 binding to telomeric DNA (27). In agreement with that study, a stoichiometry of 2 monomers per TTAGGG repeat was found by EM to fully coat >75% of the telomeric tracts on the pRST5 DNA. Preincubation of pRST5 (4 nt 5′ overhang) for 20 min at room temperature under optimal TRF1 binding conditions was followed by transcription (Materials and Methods), purification of the products and agarose gel electrophoresis. As shown (Figure 4A and B), no significant difference in the pattern of monomer and multimer bands was observed between the samples preincubated with or without either TRF1 or TRF2. EM examination did not reveal any significant effects of preincubation with either protein.

A beaded particle is frequently present at the t-loop junction and may contain TERRA

The high stability of the t-loop molecules suggested the possibility that some TERRA may remain tightly bound even following purification. To examine this, pRST5 DNA was transcribed with T7 RNA polymerase in reactions containing biotin-16-UTP. The samples were treated with RNase A and H for 1 h, deproteinized, filtered through agarose beads and then mixed with ∼50 nm paramagnetic iron particles coated with streptavidin (Materials and Methods) and filtered again to remove the unbound iron particles. Preparation for EM involved directly adsorbing the samples to thin carbon foils followed by washing, drying and rotary shadow casting with tungsten (Materials and Methods). Examination of fields of molecules showed that 40% (n = 84) of the DNA contained iron particles at one end, a percentage close to the fraction in t-loops. When the particles did not obscure the loop (Figure 5A), they were located at the t-loop junction. When two or more DNAs were joined by intermolecular HR, iron particles were bound exclusively at the DNA junctions (Figure 5B). Thus, the presence of TERRA may account in part for the high stability of the t-loop molecules. The higher resolution of tungsten shadow casting made it possible to obtain a better picture of the t-loops and junctions, and showed that the loops frequently contain a small, usually oblong bead at the loop junction (Figure 5C–G, J; and Supplementary Figure S2B–F). Measurement of the dimensions of the beads revealed an average length of 13.3 nm and width of 10.8 nm (n = 17). In experiments using T7 RNA polymerase, from 30 to 75% of the loop junctions showed these beads while the remaining junctions did not. In reactions employing T3 RNA polymerase, similar beads were observed in one experiment at 46% of the junctions (n = 154). Treatment with both RNase A and T1 (which will digest TERRA if it is not bound to DNA) did not reduce the frequency or size of the beads; however, treatment with RNase A and RNase H reduced the fraction of loop junctions with a bead by 20% and 26% in two different experiments. Nonetheless, a significant portion retained a bead. The samples had undergone extensive deproteinization with Proteinase K and SDS and thus it is unlikely that these particles represent remaining RNA polymerase. The loop junctions often contained a short DNA stem typical of regressed forks (chicken foot structures) (Figure 5I, J, Supplementary Figure S2E and F). These were more commonly found in the samples that had not undergone photo crosslinking where they were present in 17% (n = 197) of the loop junctions.

Generation of human-sized telomeric and mutant telomeric DNAs

It was important to extend these studies to telomeric DNAs approaching the size of human telomeres (3–15 kb), where the TERRA transcripts would be much longer and looping might occur over kilobase distances. Further, we wished to examine another important feature of human telomeric DNA, the block of 3 guanines in each TTAGGG repeat. In single stranded form, these blocks can participate in G quadruplex formation, a very stable structure that could

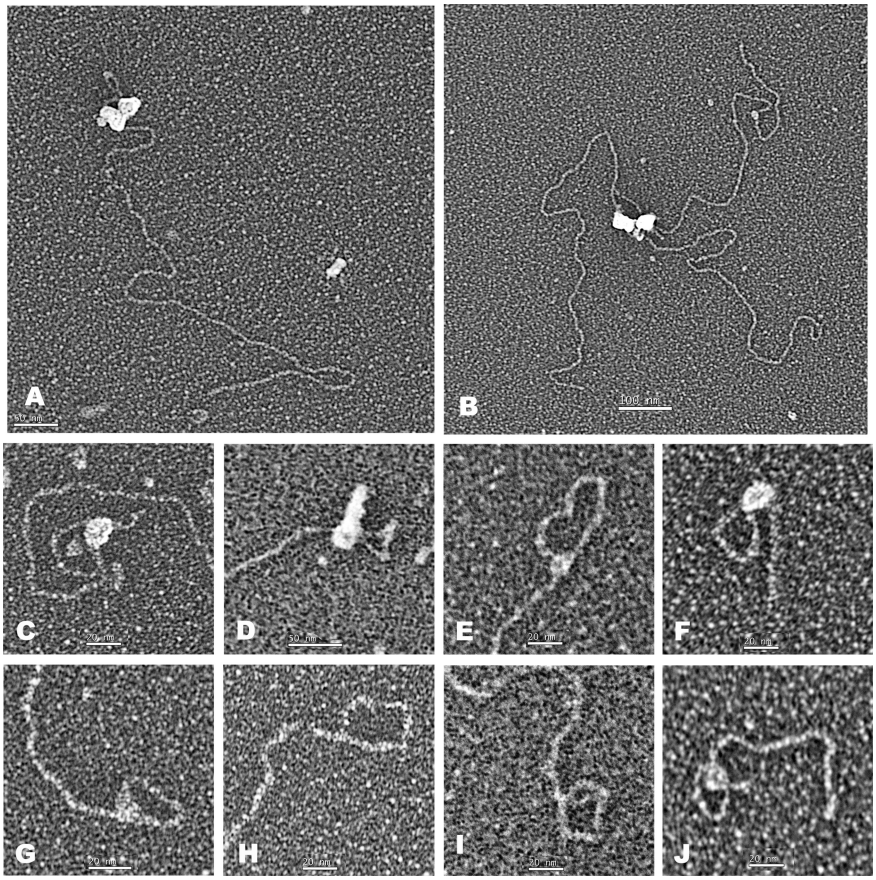

Figure 5. Architecture and composition of the telomeric tracts following transcription**.** (**A** and **B**) Linear pRST5 with no overhang or (**C**–**J**) a 54 nt 3′ overhang was transcribed for 30 min with T7 RNA polymerase in reactions containing (A and B) biotin-16-UTP or (C–J) UTP together with ATP and GTP (Materials and Methods). To detect any RNase resistant TERRA, samples were treated with RNase A and H followed by purification, and incubation with iron particles coated with streptavidin followed by removal of the unbound particles and EM preparation (text, Materials and Methods). The dense objects at the (**A**) loop junction or (**B**) junction of 3 DNAs are the iron particles. If RNase A treatment was not included, (**C** and **D**) linear molecules with loops at one end associated with a dense mass of RNA were present. (**E** and **F**): Following RNase A treatment, and purification, the loop junctions often contained a beaded particle. (**G** and **H**): Treatment of the samples with both RNase A and RNase H resulted in loop junctions, (**G**) some exhibiting a bead and (**H**) some not. (**I** and **J**) Loop junctions showing a short DNA stem extruded from the junction. Samples were mounted onto thin carbon supports followed by shadow casting with tungsten. Magnification bars are shown in each panel.

potentially contribute to the stability of the t-loop. We addressed this by generating two different mutant sequences. The first contains a single base substitution in which the middle G is replaced by thymidine. The second mutant has the same base composition as the wild-type repeat but in a scrambled order. To produce such dsDNAs with lengths that approach that of human telomeres and contain a T7 RNA polymerase promoter at one end, we used rolling circle replication of a small circular template with ϕ29 DNA polymerase and generated three different ssDNA species as long as 15 000 nt (Figure 6A), which were then converted to dsDNA (Figure 6B) with DNA polymerase I (Materials and Methods). The three ssDNAs consisted of repeating TTAGGGn, TTAGTGn in which the run of 3 G's that can form G quadruplexes is disrupted, and TGAGTGn, which has the same ratio of bases as TTAGGG but no runs of G's. The resulting DNAs ranged from a few hundred bp to some molecules approaching 40 kb, with a significant proportion in the range of 3 to 10 kb (Figure 7A–C). The 3′ end of the ds DNA opposite the T7 promoter may be blunt or may have a short 3′ G-rich ss tail depending on where the most terminal C-rich oligonucleotide annealed prior to synthesis of the complementary strand. This was verified by SSB staining and EM (not shown.)

Transcription of human-sized telomeric DNA generates t-loops and HR products

The long telomeric DNA was transcribed with either the Megascript kit or T7 RNA polymerase purified in this laboratory, which was used unless otherwise noted as it allowed us to vary known buffer components (Materials and Methods). Transcription of the wild type dsDNA and the two sequence mutants for 2 h produced abundant G-rich TERRA transcripts in equal amounts ranging in size from a few hundred nt to at least 12 000 nt as determined by agarose gel electrophoresis (Figure 6C and Supplementary Figure S3).

The long telomeric DNA (wild type sequence) was transcribed, photo crosslinked, digested with RNase A for 60 min, treated with Proteinase K and SDS, and chromatographed through agarose beads. Following preparation for EM, examination of fields revealed many of lin-

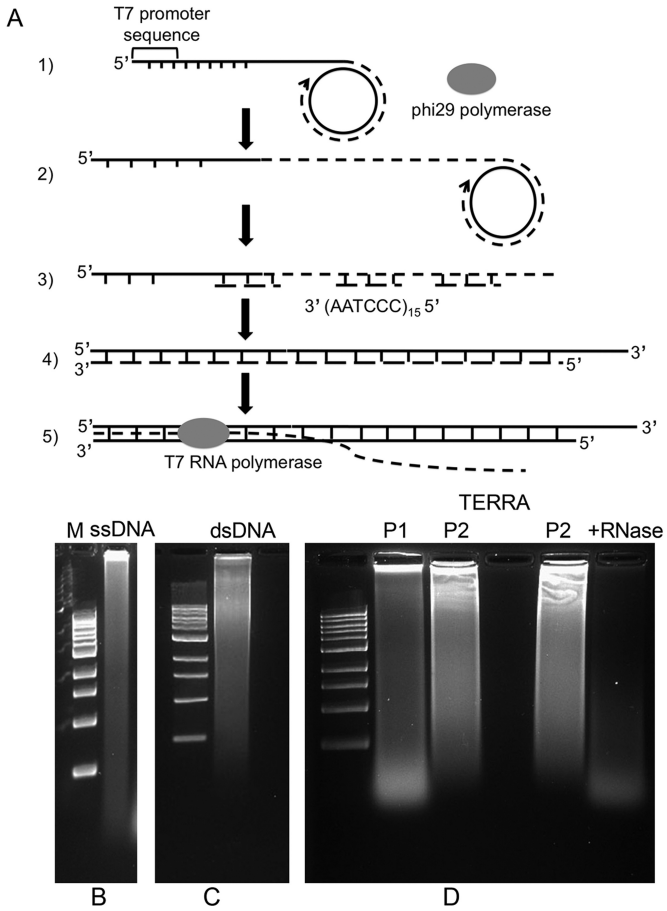

Figure 6. Agarose gel electrophoresis of long telomeric DNA and TERRA. (**A**) Schematic of the rolling circle synthesis of the long telomeric ssDNA and dsDNA. (**B**) Agarose gel electrophoresis of long G-rich ss telomeric DNA, (**C**) telomeric dsDNA and (**D**) TERRA. P1 refers to TERRA generated using the Megascript kit (Supplementary Materials) and P2 refers to TERRA generated using T7 RNA polymerase purified in this laboratory. The marker ladder consists of 1 kb steps with highest molecular weight band at 10 kb.

ear DNAs with loops at one end (Figure 7D–G and I) as well as molecules with one or more branches along their length, representing HR events between two or more DNAs (Figure 7H). In three separate experiments, 28% (57/211, 58/205, 64/223) of the long DNAs were arranged into t-loops and 23% (50/211, 47/205, 52/223) of the molecules contained one or more branches along their length (Table 1B). When the DNA was incubated for 2 h under transcription conditions but without RNA polymerase, in 3 separate experiments the percentage of DNAs with a loop at one end was ~4% (7/169, 5/111, 9/219). When T7 RNA polymerase was included but the incubation lacked NTPs, 5% (13/197, 9/174, 11/239) of the DNA were scored as t-loop forms and 3% (7/197, 5/174, 8/239) showed branches in three different experiments (Table 1B).

When the quadruplex mutant DNA $(TTAGTG)_n$ was transcribed, the looping frequency dropped to 11% (n = 385) and 8% (n = 385) of the total molecules were branched. Similarly, in reactions with DNA containing the scrambled sequence $(TGAGTG)_n$, 14% (n = 368) were looped and 10% (n = 368) were branched (Table 1B). Thus while DNAs consisting of the two mutant sequences tested here can generate looped or branched molecules upon transcription, they do so at a 2- to 3-fold lower frequency than DNA with the wild-type telomeric repeat.

In previous studies of t-loops isolated from human cells, there was no correlation between the size of the circular portion and the length of the tail, with small loops being found at the ends of long telomeric DNAs, and other, shorter telomeric fragments being mostly circular with only a short tail. This was the case here, as in several experiments employing the long DNA with the wild-type repeat the looped portions varied from 0.3 kb to 2.5 kb, with the majority being between 1 and 1.5 kb (Supplementary Figure S2). There was no clear relation between the size of the loop and the total length of the t-loop molecule.

To ask if a ss tail is required for loop formation, the long wild type telomeric DNA was digested with S1 nuclease. Agarose gel electrophoresis revealed only a modest shift to smaller lengths pointing to a relative lack of nicks or gaps in the DNA. Following S1 treatment, the long DNA was gel purified and transcribed by T7 RNA polymerase. In 3 different experiments, the percentage of DNA in t-loops was 27% (n = 243), 26% (n = 208) and 26% (n = 223), and branched forms were 25% (n = 212), 25%(n = 173) and 25%(n = 187) (Table 1B). Thus, blunt ended molecules will form t-loops upon transcription and nicks or gaps along the DNA are not required for looping.

Examination of the loop junctions formed by the long telomeric DNA revealed a fraction with small beaded particles similar to those observed with the pRST5 template (Figure 7G and I). In one experiment, 21% (n = 54) of the loop junctions showed such a bead following extensive purification with RNase A, Proteinase K and SDS. In addition, chicken foot stems were also present (Figure 7I). This observation further points to the conclusion that the loop junctions may involve a complex structure, likely containing residual TERRA.

Since the loops formed by the long (wild type) telomeric DNA were much larger than those formed on the pRST5 template, it was of interest to examine their stability. The long telomeric DNA was transcribed with T7 RNA polymerase as above but not photo crosslinked. One aliquot was then treated with only Proteinase K and SDS while another was treated with RNase A, Proteinase K and SDS. Both samples were chromatographed over agarose beads and either mounted for EM within 1 h of chromatography or allowed to remain at 4°C for 72 h before EM preparation**.** The sample treated with Proteinase K and RNase A at 1 h contained 26% loops (n = 226), whereas after 72 h at 4°C 17% (n = 219) of the molecules were in t-loops. For the aliquot not treated with RNase A, 29% (n = 253) of the DNA was in t-loops 1 h after chromatography, and after 72 h at 4°C, 21% (n = 233) of the DNA was in t-loops. Thus, the large sized transcription mediated t-loops are highly stable.

DISCUSSION

It is important to understand how transcription of telomeres impacts their global architecture, in particular the formation of intramolecular and intermolecular HR products

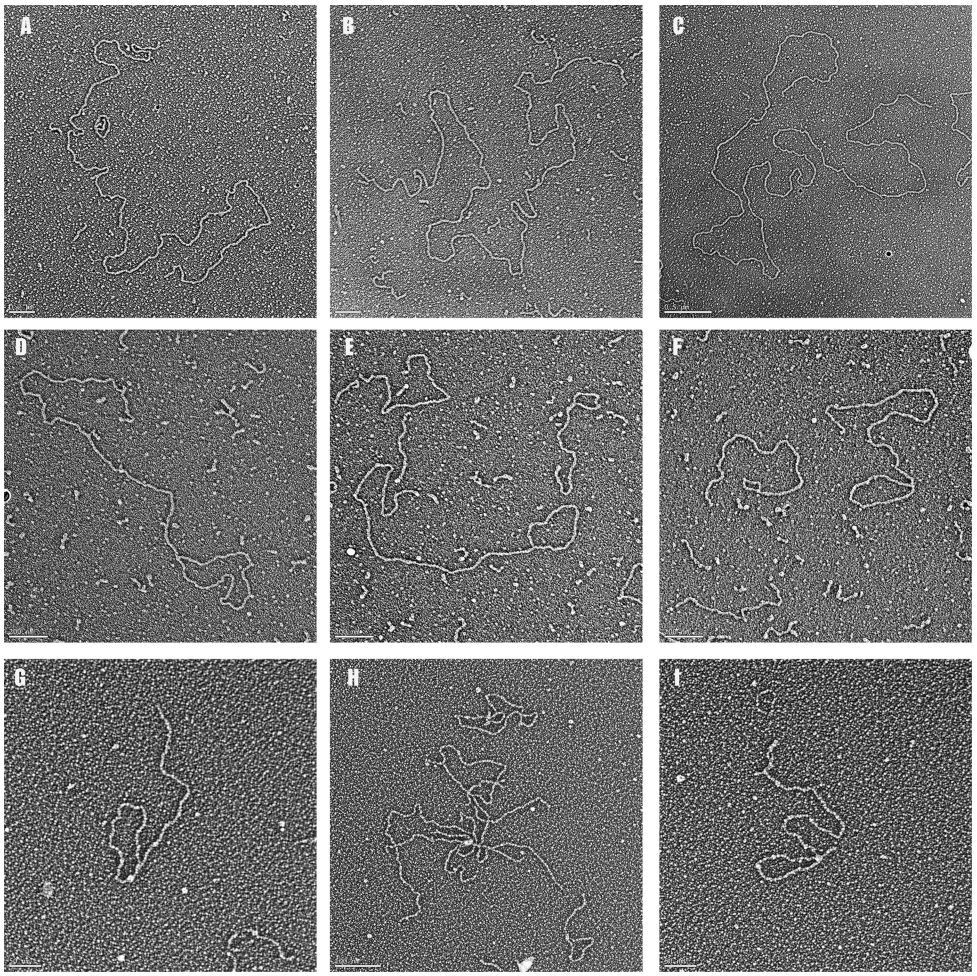

Figure 7. Visualization of long telomeric DNA and transcription products**.** Long telomeric dsDNA was generated (Figure 6, Supplementary Material) and prepared for (**A**–**C**) EM. The sizes of these very large telomeric DNAs are 21.6, 26.9 and 41.0 kb. (**D**–**G**, **I**) Following transcription with T7 RNA polymerase DNAs arranged into loops were common as well as (**H**) high branched structures. (**G**–**I**) Samples were prepared by surface spreading with cytochrome C or by adsorption to carbon supports and tungsten shadow casting. Lengths of the DNAs in D-G and I respectively are: 11.1, 8.6, 7.4, 2.2 and 2.3 kb. Magnification bars are shown in each panel.

including t-loops. Indeed, Horard and Gilson suggested that t-loops might be stabilized by TERRA (28) and Azzalin and Lingner have discussed how TERRA might be involved in telomere remodelling (29). In agreement with these authors, we observed TERRA tightly associated with the loop junctions. The work reported here describes a pathway of t-loop formation that is transcription driven rather than being dependent on TRF2.

We employed two RNA polymerases (T7, T3), which function as single polypeptides and clear their promoters at high efficiency. They are robust, readily available, and easily purified. Future studies will test reconstituted eukaryotic RNA polymerase II systems. However, this will require developing different model telomere DNAs, multiple factors will be required, and promoter clearance would be less efficient. The size of transcription bubbles is similar between the phage RNA polymerases and the eukaryotic RNA polymerase II complex (30,31). Thus, the basic changes in DNA structure induced by transcription, including formation of an R loop and/or G quartets in the displaced G-rich strand are likely to be dictated by sequence features of the DNA and not the particular RNA polymerase. Initial experiments in which the telomeric DNA was preincubated with purified TRF1 or TRF2 did not show any significant effects on t-loop formation. They were likely displaced by T7 RNA polymerase and, once removed, the subsequent events occurred as if in their absence. More detailed experiments will be warranted in the future using a eukaryotic RNA polymerase II transcription system, and the shelterins, ideally in the context of a chromatinized template.

Previous studies from this and other laboratories have pointed to a pathway in the cell for telomere looping that depends on TRF2 for either formation of t-loops or perhaps stabilization of loops once formed by a variety of mechanisms. *In vitro* studies with the pRST5 template showed that TRF2 will generate t-loops if the telomeric DNA contains a homologous 3′ G-rich ss overhang (7). Doksani *et al.* (4) then showed that functional elimination of TRF2 in the cell but not other shelterins leads to a loss of t-loops as seen by STORM imaging. This could either reflect the role of TRF2 and its partners in forming loops or, on the other hand, stabilizing loops against forces in the cell that might

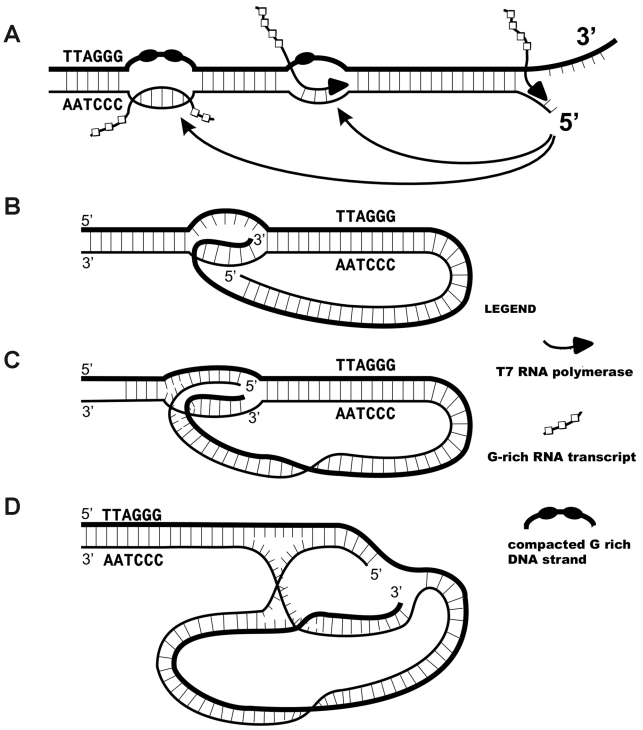

Figure 8. Models of transcription mediated telomere looping and architecture of the t-loop junctions. (**A**) A telomere undergoing transcription by RNA polymerase generates G-rich RNA arranged into chains of 24 nt beads (19). Here, a segment on the DNA following transit of the polymerase remains in an R loop with the G-rich strand compacted into G-quartet stabilized particles. When the polymerase reaches the end of the telomere the DNA strands are split apart and can pair either at the R loop or at the site of transcription. (**B**) Classic diagram of a t-loop junction in which just the long 3′ overhang has inserted into the preceding DNA to form a D-loop. (**C**) A more stable joint is generated if both DNA strands are inserted. (**D**) Illustration of the t-loop junction as a hybrid replication fork and Holliday junction. The joint in C has been drawn to illustrate its architecture in which the right side has features of a replication fork where the G-rich strand (dark strand) represents the leading strand and the C rich strand, the lagging strand. On the left, the strands cross over in manner suggestive of a Holliday junction. Migration of this crossover along the telomere repeats to the left together with pairing of the 'leading and lagging strand termini' on the right could lead to the extrusion of a regressed chicken foot structure (Figure 5I, J, Supplementary Figure S2E and F).

lead to their disruption. Given that telomere loops presumably open to allow transcription and replication, there may be multiple means for reforming and stabilizing loops.

In the cell, looping might occur between the terminus of the telomere bound by the shelterin complex including TRF2, and an internal R loop containing an extended TERRA tail which would engage the TRF2 component of the shelterin complex and bring the two sites together. Here, the primary function of the 3′ ss overhang is to position the shelterin complex at the telomere terminus poised to loop back and engage an internal R loop (Figure 8). Additional shelterin complexes bound at the R loops could further facilitate looping. For cells maintaining their telomeres by ALT, the higher levels of TERRA may allow the telomeric DNA to remain in a more open configuration peppered with R loops which would promote HR with extrachromosomal t-circles or other chromosomes.

In the diagram shown in Figure 8, a junction in which both terminal strands have invaded the preceding duplex is drawn to illustrate that these junctions have features of both a replication fork (right side) and a Holliday junction (left side). Fork regression and movement of the Holliday junction to the left would result in an extruded chicken foot stem, a possibility supported by the images shown in Figure 5I, J and Supplementary Figure S2. This complex junction could be a target for junction resolving factors, which in cells employing ALT might release t-circles. Replicative extension of the inserted 3′ terminus could extend telomere length in a telomerase-independent fashion. TRF2 and the TRF2-Rap1 complex bind Holliday junctions, suggesting a possible role in stabilizing these structures (26,32).

An unexpected feature of the loop junctions was the frequent presence of small ∼11 × 13 nm beads. Extensive deproteinization along with RNase A and RNase T treatment did not eliminate them; however, they were reduced to some extent in number by treatment with RNase H. Localization of biotin-tagged TERRA with streptavidin–iron particles showed that some residual TERRA remains at the t-loop junctions in spite of extensive RNase treatments. Thus, the beads may consist of a mixture of ss G-rich or C-rich DNA and TERRA, possibly stabilized by G-quadruplexes in a form resistant to the RNases.

Further evidence for the involvement of G quadruplexes came from the experiments with two long DNAs composed of mutants of the wild type repeat. Their transcription resulted in a 2- to 3-fold lower fraction of t-loops when compared with the wild-type repeat. The sequence changes in both disrupted the run of 3 G's capable of forming G quadruplexes. Thus, this motif appears important for either formation of loops or stabilizing them once formed. Future experiments will explore the relative stability of the loops formed by these different repeats.

Inherent in the repetitive nature of telomeric DNA, together with one strand that can form G quartets is the ability to loop back on itself upon opening of the helix by transcription. If the looping provided some benefit(s) for the stabilization and maintenance of the terminal parts of a linear genome, the ability to form t-loops may have been one of the selection forces accompanying the evolution of eukaryotic telomeric sequences and may have preceded the origin of the proteins that protect, resolve or generate t-loops themselves.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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