Tethering Telomeric Double- and Single-stranded DNA-binding Proteins Inhibits Telomere Elongation*□S

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Mammalian telomeres are composed of G-rich repetitive double-stranded (ds) DNA with a 3′ single-stranded (ss) overhang and associated proteins that together maintain chromosome end stability. Complete replication of telomeric DNA requires *de novo* elongation of the ssDNA by the enzyme telomerase, with telomeric proteins playing a key role in regulating telomerase-mediated telomere replication. In regards to the protein component of mammalian telomeres, TRF1 and TRF2 bind to the dsDNA of telomeres, whereas POT1 binds to the ssDNA portion. These three proteins are linked through either direct interactions or by the proteins TIN2 and TPP1. To determine the biological consequence of connecting telomeric dsDNA to ssDNA through a multiprotein assembly, we compared the effect of expressing TRF1 and POT1 in *trans versus* in *cis* in the form of a fusion of these two proteins, on telomere length in telomerase-positive cells. When expressed in *trans* these two proteins induced extensive telomere elongation. Fusing TRF1 to POT1 abrogated this effect, inducing mild telomere shortening, and generated looped DNA structures, as assessed by electron microscopy, consistent with the protein forming a complex with dsDNA and ssDNA. We speculate that such a protein bridge between dsDNA and ssDNA may inhibit telomerase access, promoting telomere shortening.

Telomeres are DNA-protein structures that cap and protect the ends of eukaryotic chromosomes from illegitimate recombination and degradation. In humans, the DNA portion of this structure is composed of the G-rich sequence TTAGGG repeated in tandem hundreds of times (1). This G-strand extends beyond the complementary C-strand to form a ssDNA overhang. Electron microscopy revealed that this ssDNA extension appeared to invade the dsDNA,[2] forming a D-loop, with the intervening dsDNA looping out in what is termed a T-loop. This structure has been speculated to impart some of the replicative and protective functions of telomeres (2).

In terms of the protein components of telomeres, telomeric DNA-binding proteins fall into two classes, ssDNA- or dsDNA-binding proteins. In humans, the primary dsDNA telomere-binding proteins are TRF1 (3) and TRF2 (4), whereas the principle ssDNA-binding protein is POT1 (5). Disruption of TRF2 by expression of a dominant-negative version of the protein (6, 7) and disruption of POT1 expression by RNAi or genetic knock out (8–11) can lead to various degrees of chromosome instability and/or cell arrest or death, whereas knock out of TRF1 is embryonic lethal (12). On the other hand, overexpressing these proteins alters telomere length (13–16). As access of telomerase, the enzyme that elongates the G-strand overhang of telomeres, to telomere ends is mediated by proteins in lower eukaryotes as a means of regulating telomere length; human telomere-binding proteins may also serve in this capacity (17). Thus, telomere-binding proteins can function in telomere stability and/or telomerase-mediated replication of telomeres.

Accumulating evidence argues that the ssDNA- and dsDNA-telomere-binding proteins form a complex. TRF1 and TRF2 are known to bind the protein TIN2, and TIN2 has been found to associate with another protein, TPP1, which in turn can bind to POT1 (9, 18–23). TRF2 may also associate with POT1 (9, 22). The association of these proteins has been speculated to form a protein-bridge at telomeres in which the dsDNA-binding proteins, TRF1 and TRF2, unite with the ssDNA-binding protein, POT1 (24).

The function of such a bridge remains to be fully elucidated but could very well be involved in telomere stability and replication. Although loss-of-function analysis has been an informative means to explore the role of the various components of the large telomere protein complex, each of these proteins bind to many other proteins. Hence the loss of any one protein may have a multitude of effects (23). We therefore employed gain-of-function analysis to determine the biological consequence of connecting ds- to ss-telomeric DNA through a multiprotein assembly. Specifically, we compared the effect expressing TRF1 and POT1 in *trans* on telomere length in telomerase-positive

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[2] The abbreviations used are: ds, double-stranded; ss, single-stranded; RNAi, RNA interference; PBS, phosphate-buffered saline; OB, oligonucleotide/oligosaccharide binding.

cells *versus* in *cis* whereby these two proteins are forced to constitutively interact via a direct fusion.

EXPERIMENTAL PROCEDURES

Constructs—The POT1-TRF1 fusion protein was created by inserting POT1 in frame with FLAG-TRF1 into pBabepuro (25) using a standard PCR approach. The POT1-FLAG-TRF1 cDNA was liberated by digesting with BamHI and SalI and inserted into the same sites of pBluescriptIIKS (Stratagene) for *in vitro* transcription and translation. The POT1-FLAG-TRF1 cDNA was PCR amplified, verified by direct sequencing, then blunt end ligated into the StuI site in pFastBac (Invitrogen) to make the transfer vector for bacmid preparation. pBabehygro-FLAG-TRF1 was created by subcloning FLAG-TRF1 from pcDNA3-FLAG-TRF1 into the EcoRI/SalI sites of pBabehygro (25). pBabepuro-FLAG-POT1, pCIneo-FLAG-POT1, pCIneo-FLAG-POT1ΔOB, and pEYFP-N1-Myc-TRF2 are described previously (15, 26).

Cell Culture—293T cells were stably infected with retroviruses derived from the above described pBabe plasmids and were selected with 1.0 μg/ml puromycin (Sigma) or 100 μg/ml hygromycin-B (Sigma) 48 h post-infection, as described previously (27). The first confluent plate after infection was arbitrarily assigned as population doubling 0. Following selection, cells were split, and colonies were picked to establish monoclonal cell lines.

Immunofluorescence—293T cells seeded on coverslips were transiently co-transfected with 0.9 μg of pBabepuro-POT1-FLAG-TRF1 and 0.1 μg of YFP-TRF2 with the FuGENE 6 reagent as per the manufacture's protocol (Roche). 48 h later, cells were fixed with 3.7% formaldhyde in 1× PBS for 10 min, washed twice with 1× PBS, permeabilized with 0.5% Triton X-100 in 1× PBS, washed twice with 1× PBS, and blocked with blocking buffer (1× PBS, 0.2% cold fish gelatin, 0.5% bovine serum albumin) for 30 min. The POT1-FLAG-TRF1 fusion protein was detected by incubating with anti-FLAG M2 antibody (Sigma) at a 1:5000 dilution in PBG for 1 h and recognized by incubating with donkey anti-mouse antibody conjugated with rhodamine RedX (Jackson ImmunoResearch Laboratories) diluted 1:200 in blocking buffer for 45 min. Following three 5-min washes with blocking buffer and two 5-min washes with 1× PBS, coverslips were mounted in faramount aqueous mounting medium. pEYFP-N1-Myc-TRF2 was visualized by virtue of its fluorescence. Cells were examined at ×630 magnification on an Olympus IX70 confocal fluorescent microscope.

G-strand and Double-strand Telomeric DNA Binding Assays—^{35}S-labeled proteins were synthesized *in vitro* by the T7 quick-coupled TNT system (Promega) using the plasmids pCIneo-FLAG-POT1, pCIneo-FLAG-POT1ΔOB, pRC-CMV-FLAG-TRF1, or pBluescriptIIKS-POT1-FLAG-TRF1 following the manufacturer's instructions. One-fifth of the reaction was removed to be used as an input control, and the remaining reaction mixture was diluted in 1× PBS supplemented with 0.1 mM phenylmethylsulfonyl fluoride and incubated with anti-FLAG M2-agarose affinity gel for 1 h. Resin was washed three times in 1× PBS for 5 min, and one-third of the immunoprecipitate was incubated for 30 min in binding buffer (50 mM NaCl, 25 mM Hepes, pH 7.4, 2.5 μM PBoli109 primer 5′-CCGTAAGCATTTCATTATTGGAATTCGAGCTCGT-

TTTCGA, 1 mM EDTA, pH 8.0, 100 ng/μl bovine serum albumin) containing 10 nM G-strand oligonucleotide $(T_2AG_3)_5$ or 10 nM 0.8 kbp double-stranded telomeric repeat DNA fragment, which was ^{32}P-labeled with T4 polynucleotide kinase (Invitrogen) according to the manufacturer's protocol and purified from unincorporated ^{32}P with G-25 gel filtration mini spin columns (Promega) according to the manufacturer's protocol. Unbound ss- or ds-telomeric repeat DNA was removed by washing the anti-FLAG M2 resin three times in 1× PBS for 5 min. Resin was resuspended in 1× SDS loading buffer, boiled for 10 min, and separated by electrophoresis on a SDS-PAGE gel. The gel was incubated in fixing solution (40% MeOH, 7% acetic acid, 10% glycerol) and dried. Products were visualized by exposure to a phosphorimager screen and quantified using ImageQuant version 1.0 (GE Healthcare).

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitations were performed as described previously (28) with the following modifications: a Branson sonifier microtip (Branson Ultrasonics) was used for sonication (output 3; duty cycle 30% for five 10 sec bursts), after which insoluble material was pelleted by microcentrifugation (13,000 × *g* for 5 min at 4 °C), and the remaining lysate was diluted in lysis buffer (1:2). 30 μl of 50% slurry of GammaBind G-Sepharose (Amersham Biosciences) was added to the lysate and incubated at 4 °C for 1 h to preclear the lysate. The lysate was then transferred to new tubes and immunoprecipitated overnight with anti-FLAG M2-agarose affinity gel (Sigma). Finally, dot blots were hybridized with a ^{32}P-labeled oligonulceotide telomeric probe $(T_2AG_3)_4$ in Church's buffer overnight at 50 °C followed by two washes with 4× SSC containing 0.1% SDS. After 5 days of phosphorimaging, the blots were stripped and probed with an Alu DNA repeat probe (29) in Church's buffer overnight at 42 °C followed by two washes with 2× SSC for 15 min each and two washes with 0.1× SSC containing 0.1% SDS for 10 min each. Hybridization of the probes was confirmed with 10 μg of total genomic DNA blotted on each membrane.

Telomere Length Measurements—Telomere-containing restriction fragments were visualized by resolving 10 μg of genomic DNA digested with HinfI and RsaI on 0.5% agarose gels, which were hybridized with a ^{32}P-labeled $(CCCTAA)_3$ probe followed by three washes in 15× SSC and exposed to a phosphorimager screen as described previously (30). Telomere lengths were recorded as the modal (peak) signals of the telomere-containing fragments using ImageQuant version 1.0.

Immunoprecipitations and Immunoblottings—Two 10-cm plates of the 293T cell lines were lysed in lysis buffer (1× PBS, 5 mM EDTA, 0.2% Nonidet P-40, 10% glycerol, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1.5 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4). Equal amounts of soluble lysate were incubated with 10 μl of anti-FLAG M2-agarose gel (Sigma) diluted in 100 μl of lysis buffer at room temperature for 1 h. Resin was washed twice in lysis buffer for 5 min, resuspended in 1× SDS loading dye, boiled for 10 min, resolved on SDS-PAGE gels, and immunoblotted with the anti-FLAG antibody as described previously (31).

Protein Purification—The fusion construct POT1-FLAG-TRF1 was cloned into pFastBac1 vector. The FLAG tag was positioned between the two genes. The integrity of the fusion gene was analyzed by DNA sequencing. The baculovirus was

prepared in Sf21 cells (Invitrogen) according to the manufacturer's procedures. After second amplification in SF21 cells, the virus had a titer of ~1×10^9 plaque-forming unit/ml. For purification of the fusion protein, approximately 300 ml of SF21 cells grown in suspension in Grace's Media (Invitrogen) with 10% fetal bovine serum (Sigma) were inoculated with virus at multiplicity of infection (m.o.i.) = 10. After further 48-h incubation at 27 °C, the cells were collected by spinning at 2000 rpm for 10 min. The cell pellet was washed once with ice-cold $1\times$ PBS, frozen, and kept at -80 °C till further use. The purification protocol was performed as recommended by the manufacturer (Sigma). Briefly, the cells were lysed in buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). After 30-min incubation on ice, the lysate was Dounce homogenized, sonicated, and kept on ice for 10 min more. The lysate was clarified by centrifugation at 15,000 $\times$ rpm for 30 min at 4 °C in a Sorvall SS34 rotor. 0.5 ml of pre-equilibrated with buffer A FLAG resin (Sigma) was added to the clarified extract. The purification was done as batch purification. After 2-h incubation with the resin, the resin was washed five times with Buffer A containing 300 mM NaCl. The bound protein was eluted with FLAG peptide according to manufacturer's recommendations. The purity of the protein was analyzed by SDS-PAGE and Coomassie blue staining. The POT1-FLAG-TRF1 protein was stored in aliquots at -80 °C in buffer containing 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, and 5% glycerol.

Electron Microscopy—Model telomere templates were generated as described previously (32). Briefly, pRST5 plasmid containing ~3 kb of non-telomeric DNA and a 500 bp region of telomeric repeat was linearized using BSMBI restriction site such that the 500 bp telomeric repeat tract was positioned at one end of the linearized molecule. A 54 nucleotide single-stranded overhang was created by ligation of a 58 nucleotide telomeric oligonucleotide onto the telomeric end. The model telomere templates were incubated in a reaction with 100 ng each of TRF1 and POT1-TRF1 for 30 min on ice, in buffer containing 20 mM HEPES, 80 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA. Samples were fixed with 0.6% glutaraldehyde, and protein-DNA complexes were isolated over a 2.5-ml Bio-Gel A 15M column and incubated with spermidine before they were directly adsorbed to glow-charged carbon foil grids, dehydrated by a series of water and ethanol washes and rotary shadow cast with tungsten. Images were collected using an FEI Tecnai 12 electron microscope and Gatan Ultrascan US400SP digital camera with Gatan Digital Micrograph software.

RESULTS AND DISCUSSION

The POT1-TRF1 Fusion Protein Exhibits Telomeric dsDNA and ssDNA Binding Activities—Large complexes containing both the telomeric ssDNA-binding protein POT1 and telomeric dsDNA-binding proteins TRF1 and TRF2 have been isolated from mammalian cells (20, 22), although the biological importance of combining these two different DNA-binding activities in one complex is unclear. To this end, we used a gain-of-function approach to determine the effect on telomere length in telomerase-positive cells upon physically tethering the telomeric ssDNA-binding protein POT1 to the telomeric dsDNA-binding protein TRF1, such that the telomeric dsDNA-

and ssDNA-binding activities would be constitutively united, compared with expressing TRF1 and POT1 in *trans*.

To begin this analysis, we first created a POT1-TRF1 telomere chimeric protein by fusing the human POT1 cDNA in-frame to the N terminus of human TRF1, with the FLAG epitope sequence acting as a linker peptide between the two proteins. In this configuration, the DNA-binding domains of both proteins were at the extreme termini of the fusion protein to minimize negative effects on their activity (Fig. 1*A*).

We next tested whether the fusion protein retained the ability to bind telomeric dsDNA and ssDNA *in vitro*. Specifically, to test for ss-telomere binding, ^{35}S-labeled recombinant POT1-TRF1 fusion protein was generated in rabbit reticulocyte lysate *in vitro* and incubated with a ^{32}P-labeled G-strand telomere oligonucleotide. The fusion protein was immunoprecipitated by virtue of the FLAG epitope tag and resolved by SDS-PAGE followed by exposure to film to visualize the labeled protein and DNA, as described previously (5, 15). As negative controls we similarly assayed a POT1 protein lacking one of the telomeric ssDNA-binding OB-fold domains (POT1ΔOB) and wild-type TRF1 (that binds telomeric dsDNA and not ssDNA) and as a positive control, POT1. As expected, the positive-control POT1 co-immunoprecipitated with the telomeric oligonucleotide. Similarly, although the fusion protein was expressed at lower levels, it clearly co-immunoprecipitated with telomeric ssDNA. This association depended upon the DNA-binding activity of POT1, as neither the POT1ΔOB nor the TRF1 protein co-immunoprecipitated with the same DNA (Fig. 1*B*). Thus, the fusion protein bound telomeric ssDNA *in vitro*.

To determine whether the fusion protein retained the ability to bind telomeric dsDNA, POT1-TRF1, positive control TRF1, or negative control POT1 (that binds only telomeric ssDNA), ^{35}S-labeled recombinant proteins were mixed with ^{32}P-labeled telomeric dsDNA, immunoprecipitated, and resolved by SDS-PAGE, akin to the method used to monitor POT1 binding to telomeric ssDNA. Positive-control TRF1 and the fusion protein both co-immunoprecipitated with the telomeric dsDNA, whereas negative control POT1 protein did not. Thus, the fusion protein can also bind telomeric dsDNA *in vitro* (Fig. 1*C*). The presence of truncated products that could also bind DNA precluded determining the degree that the full-length fusion protein associated with DNA. Taken together, we conclude that the fusion protein retained the ability to bind both telomeric ssDNA and dsDNA *in vitro*.

The POT1-TRF1 Fusion Protein Localizes to Telomeres in Vivo—We next extended these results *in vivo* by assaying whether this fusion protein could also localize to the nucleus and associate with telomeres. POT1-TRF1 was transiently co-expressed in 293T cells with a YPF-tagged version of the telomere-binding protein TRF2 to mark telomeres (4). POT1-TRF1, as detected by immunofluorescence via the FLAG epitope, formed punctate staining within the nucleus. YFP-TRF2 similarly formed nuclear foci indicative of telomeres, as detected via the fluorescence of YPF, and these foci co-localized with POT1-TRF1 (Fig. 1*D*). Telomeric DNA association with POT1-TRF1 was verified by an independent *in vivo* assay, namely chromatin immunoprecipitation. Specifically, the fusion protein co-immunoprecipitated with telomeric DNA, as
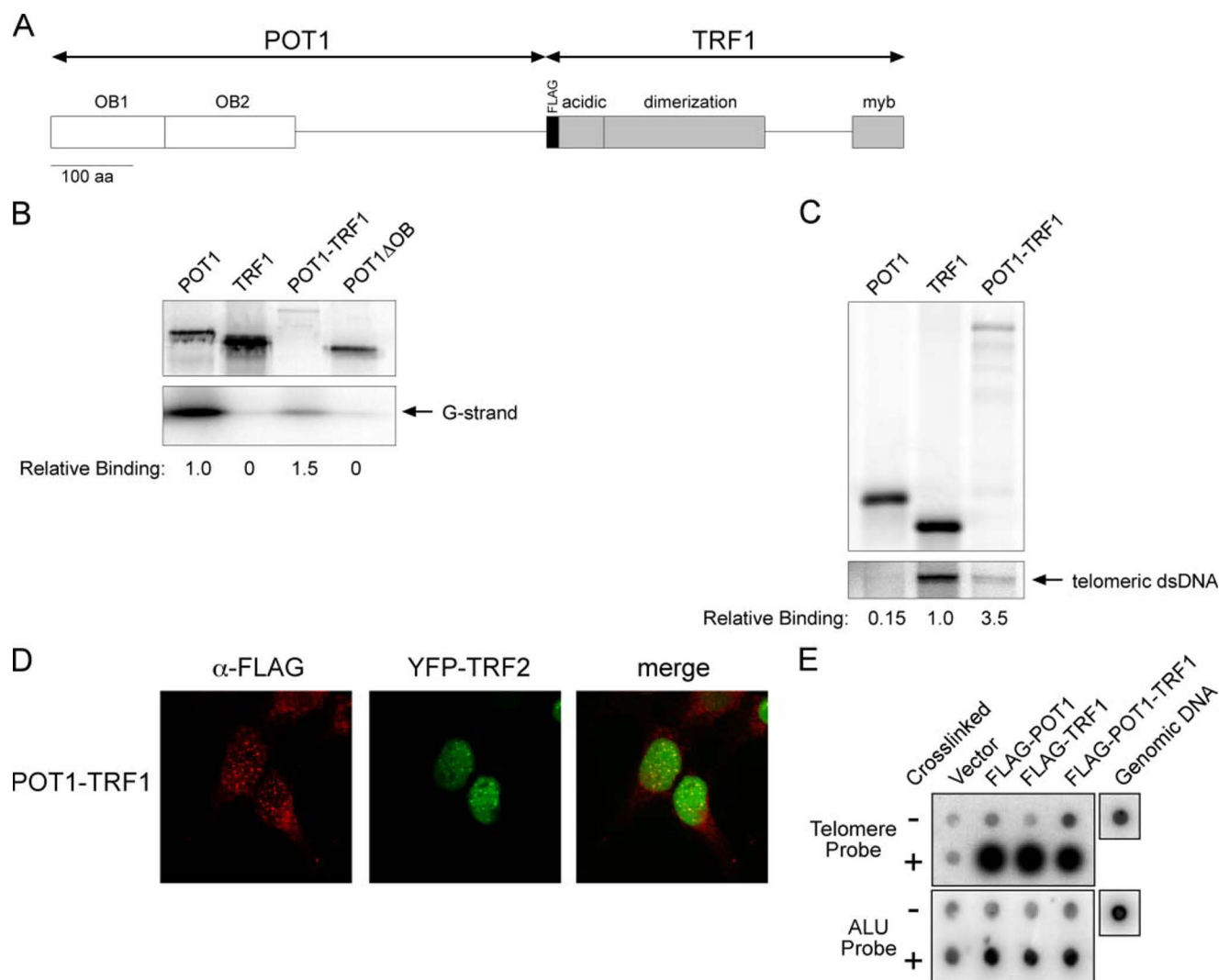
FIGURE 1. **POT1-TRF1 binds to telomeres.** *A*, scale diagram of the POT1-TRF1 fusion protein. *OB1* and *OB2*, oligonucleotide/oligosaccharide binding (OB)-fold telomeric ssDNA-binding domains; *FLAG*, FLAG epitope tag used as a linker; *acidic*, acidic domain, dimerization; *TRF1*, dimerization domain; *myb*, myb-type DNA binding motif. *B* and *C*, POT1-TRF1 fusion protein binds telomeric DNA *in vitro*. Immunoprecipitation of *in vitro* produced ^{35}S-labeled FLAG-tagged proteins incubated with ^{32}P-labeled G-strand oligonucleotide (*B*) or ^{32}P-labeled telomeric dsDNA fragment (*C*). *Bottom panel*, binding of DNA relative to protein expression. *D*, POT1-TRF1 fusion protein co-localizes with the telomere-binding protein TRF2. An example of 293T cells transiently co-expressing YFP-TRF2 and POT1- TRF1 stained with an α-FLAG antibody to detect the POT1-TRF1 fusion protein (*left panel*), viewed as a fluorescence image to detect YFP-TRF2 (*middle panel*), or a merge of both images (*right panel*). *E,* 293T cells stably expressing FLAG-POT1, FLAG-TRF1, FLAG-POT1-TRF1, or empty pBabe vector were treated with formaldehyde to cross-link proteins and DNA or left untreated as a control. The cells were then subjected to chromatin immunoprecipitation with α-FLAG-agarose affinity gel followed by Southern blotting with a telomeric probe. As a control, membranes were also stripped and re-hybridized with an Alu probe to determine nonspecific DNA interactions. Hybridization of the probes was confirmed using genomic DNA.

assessed with a telomeric DNA probe, and moreover, this association was abolished if DNA and protein were not cross-linked. The fusion protein bound specifically to telomeric DNA, and not irrelevant DNA (detected with an Alu DNA probe), and to a similar level as positive control POT1 and TRF1 proteins (Fig. 1*E*). Taken together, these data argue that the fusion protein is able to associate with telomeres *in vivo*.

The POT1-TRF1 Fusion Protein Induces Lariat DNA Structures—To explore the effect of expressing POT1-TRF1 on telomeric DNA structure, the fusion protein was highly purified from insect cells (Fig. 2*B*) and incubated with telomeric DNA in a 1:1 ratio with purified TRF1. We included TRF1 in these reactions because this protein must dimerize to bind telomeric DNA, and in cells endogenous TRF1 presumably complexed with the fusion protein (3, 33). Indeed, TRF1 was found to foster

the binding of the fusion protein to telomeric dsDNA (not shown). The model telomere substrate was composed of ~3 kb of non-telomeric DNA ending in 500 bp of telomeric dsDNA with a 54 nucleotide 3′ overhang.

Three types of DNA-protein complexes were noted. First, consistent with the ssDNA-binding activity of POT1 (5), 47.8% ± 11.1 of the DNA-protein complexes had protein at the end (ssDNA) of the substrate. Second, consistent with the known dsDNA-binding activity of TRF1 (3, 33), 15% ± 3.3 of the DNA-protein complexes had protein bound internally within the 500 bp telomeric dsDNA region of the substrate.

Third and most interesting, 12.8% ± 4.4 of the DNA-protein complexes were characterized by a protein complex at the junction of a lariat DNA structure. This latter structure was depend-
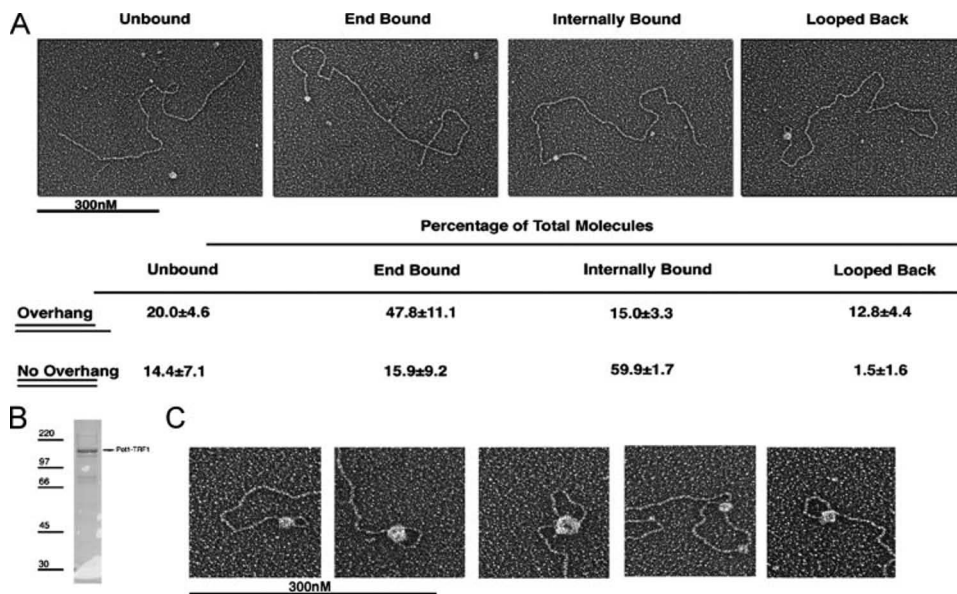
FIGURE 2. *In Vitro* **binding and remodeling of synthetic telomeres by co-incubation of purified TRF1-POT1 fusion protein and TRF1.** The TRF1-POT1 fusion protein and TRF1 were purified in insect cells and co-incubated with model telomeres according to the conditions in the text. Protein-DNA complexes were fixed, isolated over an A5M BioGel column, followed by adsorption onto glow-charged copper grids and rotary shadowcast with tungsten. *Images* are shown in reverse contrast. *A, top panel*, examples of molecules generated on model telomeres 500 bp ending in a 54 nucleotide single-stranded overhang of TTAGGG. *Bottom panel*, model telomeres 500 bp were generated with or without the 54 nucleotide overhang and incubated with both the TRF1-POT1 fusion protein and TRF1. DNA molecules were classified as unbound, protein-bound at one end, proteins internally bound along the telomere tract, or arranged into loops. More than 560 molecules were scored in three independent experiments, and the percentage of molecules in each category was determined. Values represent the average number of molecules in each category ± S.E. *B*, higher magnification of loops formed by TRF1-POT1 fusion protein and TRF1 on model telomeres 500 bp.

formed cell line 293T, a subline of the telomerase-positive cell line 293 (30), was stably infected with a retrovirus encoding the POT1 and TRF1, POT1-TRF1, or no transgene (vector). Previously it had been shown that clonal populations could serve as a better indicator than polyclonal populations of the variability on telomere length when overexpressing a telomere-binding protein (16). Therefore, we picked and cultured 13 clones from cells expressing POT1 and TRF1 in *trans*, 14 clones expressing these proteins in *cis* (POT1-TRF1), and 12 clones from vector cells (Fig. 3, *A–C*), confirmed expression of the indicated proteins by immunoblot analysis (Fig. 3, *D–F*), and measured their telomere length by Southern hybridization after an average of 35–40 population doublings.

As previously reported, telomere length can vary in clonal populations expressing telomere-binding proteins (16), and indeed this was observed in all the clones (Fig. 3, *A–C*) and also polyclonal populations (supplemental Fig. 1 and not shown) from the three different genotypes. This variation was apparently not a product of differential ectopic expression of POT1, TRF1, the fusion POT1-TRF1 protein, or a truncated version thereof (Fig. 3, *E* and *F*). Despite this variability, co-expression of POT1 and TRF1 in *trans* promoted telomere elongation. Specifically, the average modal telomere length of the 13 clones expressing POT1 and TRF1 in *trans* was 12.7 kbp, with some clones having an average telomere length as high as 17.2 kbp (Fig. 3*B*). On the other hand, the modal telomere length of vector cells ranged from 4.5 to 8.7 kbp, with an average length of the 12 clones being 6.9 kbp (Fig. 3*A*). Similar results were found also with polyclonal populations (supplemental Fig. 1). Thus, telomeres were elongated by an average of 5.3 kbp in cells expressing POT1 and TRF1 in *trans*.

In contrast, cells in which POT1 and TRF1 were engineered to constitutively interact via a direct fusion had an average telomere length of 6.2 kbp (Fig. 3*C*), a full 6.5 kbp shorter on average than when POT1 and TRF1 were expressed in *trans* (Fig. 3*B*). Although there was again telomere length heterogeneity among the 14 clones (Fig. 3*C*) as well as polyclonal populations (supplemental Fig. 1 and not shown) expressing POT1 and TRF1 in *cis*, all these clonal and polyclonal populations had telomeres shorter than the average telomere length of cells expressing these proteins in *trans*, with the most extreme difference being 15.9 kbp between the two genotypes. The fusion protein not only negated the effect of overexpressing POT1 and TRF1, it also induced mild telomere shortening compared with vector-infected cells. Cells expressing the fusion protein had an

ent upon the telomeric ssDNA. Specifically, when the experiment was repeated using a similar model telomere substrate composed of 500 bp of telomeric dsDNA, but lacking the 3′ overhang, there was a shift in the complexes from those with protein at the end or in a lariat, to almost exclusively complexes with protein bound within the telomere repeat tract (Fig. 2, *A* and *C*). These results were highly reproducible, as they were generated from three independent experiments in which over 560 molecules were scored. TRF1 alone does not induce these structures (2). POT1 could not be purified to similarly test, and hence it is formally possible that POT1 alone may induce such structures. Arguing against this possibility is the fact that POT1 binds only ssDNA (Ref. 5 and Fig. 1, *A* and *C*), but the loops are presumably a union of dsDNA and ssDNA because of their large size. Taken together, we suggest that either the fusion protein tethers both dsDNA and ssDNA together, looping out the intermediate DNA, or that this protein complex stabilizes the structure formed when the ssDNA invades the dsDNA and again loops out the intervening DNA (2). In either scenario, the fusion protein fosters a complex composed of telomeric dsDNA and ssDNA.

Expression of POT1 and TRF1 in cis versus in trans Causes Opposite Effects on Telomere Length—Given that the fusion protein retained the DNA-binding activities of POT1 and TRF1 and could foster the union of telomeric dsDNA and ssDNA, we next addressed what effect expression of POT1 and TRF1 in *trans versus* in *cis* in the form of a fusion protein would have on telomere length in telomerase-positive cells. The virally trans-
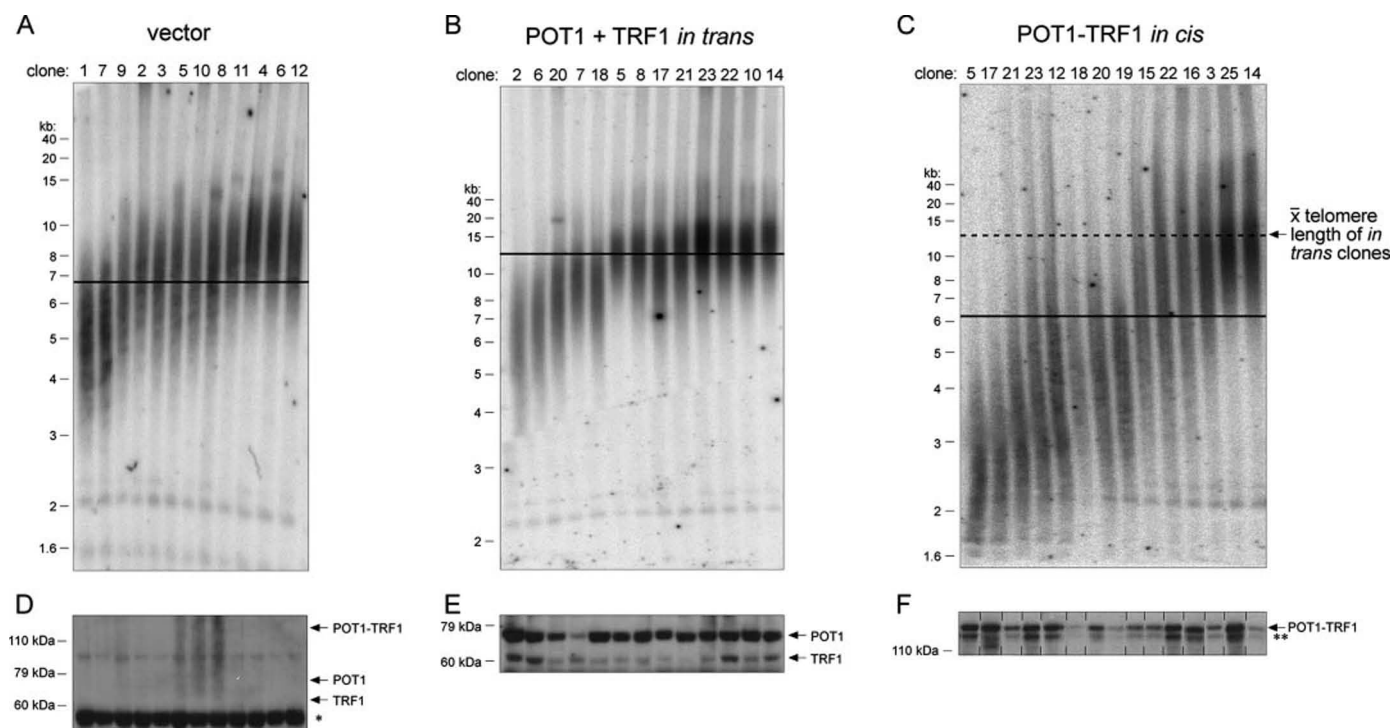
FIGURE 3. **The effect of expressing POT1 and TRF1 in *trans versus* in *cis* on telomere length.** *A–C,* genomic DNA isolated at an average of ~40 population doublings from monoclonal cell lines derived from 293T cells expressing (*A*), no transgene (*vector*) (*B*), POT1 and TRF1 in *trans* or (*C*), the POT1-TRF1 fusion protein in *cis* were assayed for telomere length by Southern blotting using a telomeric probe. *Line* indicates the approximate average telomere length of clones for each condition. *Dashed line* indicates the approximate average telomere length of POT1 + TRF1 for comparison purposes. *D–F,* equal amounts of total protein isolated from the indicated monoclonal cell lines were subjected to immunoprecipitation with α-FLAG M2 resin and immunoblotted with α-FLAG M2 antibody to detect POT1, TRF1, and the POT1-TRF1 fusion protein. The expected sizes of proteins are indicated. *, antibody heavy chain in vector cells; **, a truncation product of the POT1-TRF1 fusion protein.

average telomere length almost 1 kbp shorter than vector cells, reflecting the observation that two thirds of the clones had telomeres shorter than vector cells. Moreover, vector cells (Fig. 3*A*) never exhibited telomeres as short (2.3 kbp) as those seen in the fusion-expressing cells (Fig. 3*C*).

Summary—To explore the biological consequence of a complex containing both telomeric dsDNA- and ssDNA-binding proteins, we compared the effect on telomere length of expressing POT1 and TRF1 in *trans versus* in *cis* via a direct fusion of these proteins to generate a chimeric protein capable of binding telomeric dsDNA and ssDNA *in vitro*. We report that in *trans* these proteins induced extensive telomere elongation, whereas in *cis* this effect was abrogated, and if anything, led to telomere shortening in many of the clones. Not all clones exhibited as dramatic difference in length, consistent with the previous observation that complex telomere patterns are seen when POT1 or other telomere-binding proteins are overexpressed (14, 16). Although a number of mechanisms by which this fusion protein may act oppositely compared with co-expression of TRF1 and POT1 are possible, including fusion of these proteins inhibiting some function of TRF1 or POT1 aside from DNA binding, some insight was provided by electron microscopic analysis of complexes formed with the fusion protein and a telomeric substrate. This protein fostered the production or stabilization of lariat structures, in which dsDNA and ssDNA formed a complex with the proteins. We speculate that such a structure may resist access of telomerase and correspondingly may promote the telomere shortening

observed in cells. In support of this model, knocking down the expression of some proteins in the telomeric complex has been reported to cause the opposite phenotype to fusing the telomere proteins, namely telomere elongation (24). Collectively, these data support a model whereby a complex between telomeric dsDNA- and ssDNA-binding proteins negatively regulate telomere length.

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