TRF1 Inhibits Telomere C-Strand DNA Synthesis in Vitro†

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ABSTRACT: Human TTAGGG repeat-binding factor 1 (TRF1) is involved in the regulation of telomere length in vivo, but the mechanism of regulation remains largely undefined. We have developed an in vitro system for assessing the effect of TRF1 on DNA synthesis using purified proteins and synthetic DNA substrates. Results reveal that TRF1, when bound to telomeric duplex DNA, inhibits DNA synthesis catalyzed by DNA polymerase α/primase (pol α). Inhibition required that TRF1 be bound to duplex telomeric DNA as no effect of TRF1 was observed on nontelomeric, random DNA substrates. Inhibition was shown to be dependent on TRF1 concentration and the length of the telomeric duplex region of the DNA substrate. When bound in cis to telomeric duplex DNA, TRF1 was also capable of inhibiting pol α-catalyzed DNA synthesis on nontelomeric DNA sequences from positions both upstream and downstream of the extending polymerase. Inhibition of DNA synthesis was shown to be specific for TRF1 but not necessarily for the DNA polymerase used in the extension reaction. In a series of control experiments, we assessed T7 DNA polymerase-catalyzed synthesis on a DNA template containing tandem gal4 operators. In these experiments, the addition of the purified Gal4-DNA binding domain (Gal4-DBD) protein has no effect on the ability of T7 polymerase to copy the DNA template. Interestingly, TRF1 inhibition was observed on telomeric DNA substrates using T7 DNA polymerase. These results suggest that TRF1, when bound to duplex telomeric DNA, serves to block extension by DNA polymerases. These results are discussed with respect to the role of TRF1 in telomere length regulation.

Telomeres, the repetitive DNA sequences found at the ends of eukaryotic chromosomes, serve to protect chromosomes from degradation, prevent chromosome fusions (*1*), and have been shown to be involved with chromosome segregation during meiosis (*1*, *2*). In addition, telomere repeats can serve as a buffer to protect the coding and regulatory DNA against their loss as a result of the end replication problem (*3*). This gradual chromosome shortening is observed in most somatic cells and eventually leads to cellular senescence (*4*). Complete telomere replication provides a mechanism whereby chromosome length can be maintained. The major pathway for telomere maintenance involves the enzyme telomerase (*5*), although alternative mechanisms have been identified in cells lacking telomerase (*6*, *7*). Telomerase participates in the pathway to prevent chromosome shortening by acting after replication (*8*) or following any other mechanism by which telomeres are shortened. After DNA replication, removal of the terminal RNA primer from the lagging strand results in a 3′ overhang of uncopied DNA. This intermediate can serve as a substrate for telomerase. Telomerase extends the 3′ overhang using an internal RNA template to synthesize multiple d(TTAGGG) repeats, creating a lengthened G-rich single strand (*5*). The action of telomerase is capable of only half of the telomere maintenance pathway, whereas complete telomere maintenance requires synthesis of the complementary C-strand. Considerable in vitro (*9*) and in vivo (*10*−*13*) evidence suggests that DNA pol α/primase is an integral component of the telomere C-strand synthesis pathway.

In any given clonal cell line, telomere length is relatively constant and independent of chromosome size (*13*). A series of elegant studies have demonstrated that the regulation of telomere length is determined by the occupancy of telomere DNA by duplex telomere DNA binding proteins. This effect was first observed in budding yeast (*14*) and subsequently in fission yeast and mammalian cells (*15*−*18*). Human chromosomes have two known telomere-associated proteins termed human TTAGGG repeat-binding factors 1 and 2 (TRF1[1] and TRF2, respectively) (*15*). There are also many other proteins that have been found to associate with telomeres, a number of which are involved in length regulation and telomere maintenance (*19*−*21*). TRF1 has been shown to affect telomere maintenance in telomerase positive cells (*18*). Specifically, overexpression of TRF1 resulted in a decrease in telomere length, while overexpression of a dominant negative TRF1 mutant was accompanied by an increase in telomere length. While these results implicate TRF1 in telomere length regulation, the exact mechanism of TRF1 regulation has not yet been described. Indirect evidence suggests that TRF1 inhibits telomere lengthening by directly inhibiting telomerase (*22*). However, these reports provide no direct evidence that telomerase is the target of TRF1 inhibition.

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[1] Abbreviations: TRF, TTAGGG repeat-binding factor; pol, polymerase; Gal4-DBD, Gal4-DNA binding domain.

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The data presented in this paper demonstrate that TRF1, when bound to duplex telomeric DNA, can inhibit DNA polymerase-catalyzed DNA synthesis. Inhibition is specific for TRF1 but not the polymerase catalyzing synthesis. These data are discussed with respect to the effect of TRF1 on DNA structure, and the role of pol α and TRF1 in telomere length regulation.

MATERIALS AND METHODS

Protein Purification. Pol α/primase was purified from calf thymus as described previously (*9*). TRF1 was purified from Sf21 insect cells following infection with a recombinant baculovirus expressing a His_6-tagged TRF1 fusion protein (*23*). Briefly, 1×10^8 Sf21 cells were infected at an MOI of 10 and collected 48 h postinfection. The cell pellet was washed with PBS and suspended in buffer R [50 mM NaP_i (pH 7.5), 1 M KCl, 0.2% Triton X-100, 7 mM β-mercaptoethanol, and 10% glycerol] supplemented with 5 mM imidazole. The cells were disrupted by dounce homogenization, and DNA was fragmented by sonication. Polyethyleneimine was added to a final concentration of 0.1% and sedimented at 10000*g* for 10 min. The supernatant was adsorbed to 4 mL of phosphocellulose equilibrated in the same buffer. The mix was poured into a column, and the flow-through was collected and clarified by sedimentation at 10000*g* for 15 min. The clarified supernatant was applied to a 2 mL nickel−NTA agarose column (Qiagen) equilibrated with the same buffer. The column was washed with the same buffer, and TRF1 eluted with buffer R supplemented with 350 mM imidazole. TRF1-containing fractions were identified by Coomassie blue-stained SDS−PAGE analysis of fractions. The eluant from the Ni−NTA column was pooled and diluted with buffer A [50 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, and 10% glycerol] to a KCl concentration of 100 mM. The diluted protein was applied to a 2 mL heparin−Sepharose column equilibrated with buffer A and 0.1 M NaCl. The column was washed and bound protein eluted with a 20 mL linear gradient from 0.1 to 1 M NaCl in buffer A. TRF1-containing fractions were identified, pooled, and dialyzed versus buffer D [20 mM Hepes (pH 7.5), 1 mM β-mercaptoethanol, 0.5 M KCl, and 20% glycerol]. The plasmid pHisGal, encoding a N-terminal His_6-tagged Gal4-DBD, was obtained from ATCC. The plasmid was transformed into *Escherichia coli* XL-1 Blue, and a single colony was grown to an A_{600} of 0.8. Protein expression was induced with 1 mM IPTG for 4 h, and the cells were then collected and lysed by sonication. The Gal4-DBD was purified by sequential chromatography on Ni−agarose and heparin−Sepharose matrixes. The final yield was 1.5 mg from a 500 mL culture. Coomassie blue-stained SDS gel analysis of the final protein preparations for TRF1 and Gal4-DBD is presented in Figure 1. Each protein was greater than 90% pure as judged by the SDS gel analysis.

DNA Substrates and Enzymatic Assays. The DNA oligonucleotides used to construct the DNA substrates are presented in Table 1. The individual DNA substrates used in this study are depicted in Figure 2. Partial duplex DNA substrates were prepared by annealing the required oligonucleotides at a primer:template ratio of 2:1. DNA synthesis reactions were performed in 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 7.5 mM DTT, supplemented with dATP, dTTP, and dGTP (each at 100 μM) and [α-^{32}P]dCTP (5 μCi, 20 μM), and the mixtures contained 0.5 pmol of template/primer DNA substrate unless noted otherwise. For assays using single-stranded DNA substrates requiring primase activity, the reaction mixtures contained 5 pmol of template DNA and were supplemented with ATP, UTP, GTP, and CTP (each at 1 mM) (*9*). Reaction mixtures were incubated for 30 min at 25 °C unless indicated otherwise, and reactions were terminated by the addition of EDTA to a final concentration of 20 mM. Unincorporated nucleotides were removed by Sephadex G-50 spin column chromatography, and products were collected by ethanol precipitation. Products were resuspended in 50% formamide and 5 mM EDTA, heat denatured at 95° C for 5 min, and separated on 8 or 10% polyacrylamide/7 M urea DNA sequencing gels. The gels were dried under vacuum, and products were visualized by autoradiography and quantified by PhosphorImager analysis.

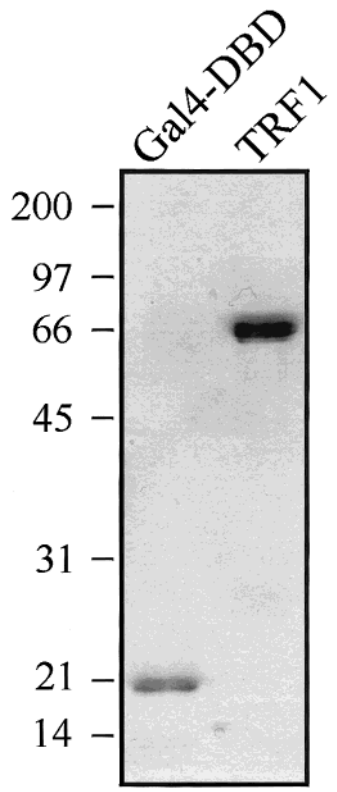

FIGURE 1: Purification of TRF1 and Gal4-DBD. The final pool of TRF1 produced in insect cells and Gal4-DBD produced in *E. coli* were separated by SDS−PAGE. The gel was stained with Coomassie blue and indicates that each protein is greater than 90% pure. TRF1 migrates as a 66 kDa protein and Gal4-DBD at a position equivalent to 21 kDa.

RESULTS

TRF1 serves an important function in the regulation of telomere length. The protein counting model for telomere length regulation describes a mechanism where the length of telomeric DNA repeats is measured so that once the requisite number of duplex telomere DNA binding proteins is bound, further elongation is inhibited (*24*). The direct mechanism of how telomere-bound duplex DNA binding proteins affect the telomere replication machinery remains to be determined. We previously established an in vitro assay whereby DNA pol α/primase is capable of priming and copying a telomeric G-strand template (*9*). Using this assay,

Table 1

oligo	length (nucleotides)	DNA sequence (5′ to 3′)
TS10	60	$(TTAGGG)_{10}$
TS8+	67	$(TTAGGG)_8$TGATCTCCCTTTCCTTCTT
PS59	59	ATCGCCTGAGTCAGAGCTAGCTAGCCCAGGATCCACCGAATGATCCCCT TTCCTTCTT
PSC18	18	AGAAGGAAAGGGAGATC
UST	81	ATCGGCTGAGTCAGAGCTAGCTAGCCCAGGATCCACCGAATCAGGATGCT GAAGCCA$(TTAGGG)_4$
DST	99	CAGGATGCTGAAGCCT$(TTAGGG)_4$ATGCGCTGAGTCAGAGCTAGCTAGCCC AGGATCCACCGAATGATCTCCCTTTCCTTCTT
DSP	40	$(CCCTAA)_4$TGGCTTCAGCATCCTG
4gal4+	106	GTCGGAGTACTGTCCTCCGACTGTCGGAGTACTGTCCTCCGACTGTCGGAGT- ACTGTCCTCCGACTGTCGGAGTACTGTCCTCCGACTGATCTCCCTTTCCTTCTT

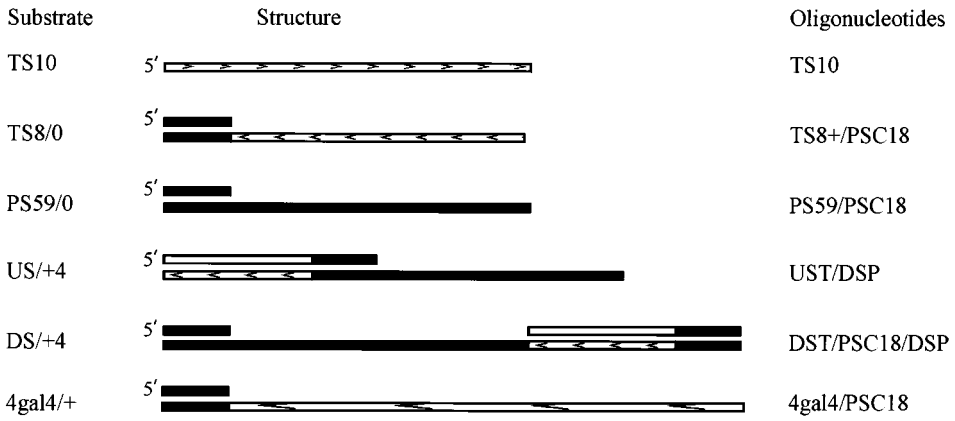

FIGURE 2: DNA substrates used in this study. The substrate name is presented to the left of the cartoon depicting the structure of each DNA substrate. The filled lines represent nontelomeric DNA, and the open boxes with arrows represent telomeric DNA repeats in the 5′ to 3′ direction on the G-rich strand. The number of arrows indicates the number of telomeric repeats. The arrows in the 4gal4/0 substrate represent gal4 operator sequences. The individual oligonucleotides used to prepare the DNA substrates are indicated to the right of each structure.

we set out to test the hypothesis that TRF1 regulation of telomere length occurs at the level of telomere C-strand synthesis. Therefore, we assessed the effect of purified TRF1 on telomere C-strand synthesis catalyzed by pol α/primase. Priming and copying of a synthetic 60-base substrate, TS10, was assessed in reactions performed in the absence or presence of increasing concentrations of TRF1. The results shown in Figure 3 demonstrate that in the absence of TRF1, pol α is capable of priming and copying the TS10 substrate, resulting in a distribution of products at six-base intervals consistent with site specific priming opposite the two thymidines, as we have previously described (*9*). The addition of TRF1 resulted in a concentration-dependent decrease in the amount of synthetic products with a greater degree of inhibition observed for the longer products. Quantification of the data (not shown) reveals greater than 85% inhibition of the 60-base product, 60% inhibition of synthesis of the 40-base product, and 40% inhibition of synthesis of the 22-base product. These data demonstrate a length dependence of TRF1 inhibition where a TRF1 binding site of 24−36 bp is required before significant inhibition of C-strand synthesis is observed and suggest a mechanism whereby TRF1 binds to the telomeric duplex regions of the substrate and inhibits further extension of the C-strand. An alternative possibility is that TRF1 preferentially inhibits priming by pol α at the 3′ end of the TS10 template DNA, which would also be manifested as a decrease in the amount of longer products. To distinguish between these two alternatives, a DNA substrate, TS8/0, was prepared consisting of eight d(TTAGGG) repeats followed by a 3′ 18-base nontelomeric region. Annealing an 18-base primer complementary to the nontelomeric region results in a primer−template substrate capable of being extended by DNA pol α to copy the telomere G-strand independent of primase activity. These reactions were performed in the absence of rNTPs to exclude the possibility of RNA primer synthesis at the telomeric sequences; therefore, all products that are observed are by extension of the 18-base primer. The results shown in Figure 4B demonstrate that TRF1 is able to inhibit C-strand synthesis in the absence of primase activity; thus, inhibition by TRF1 is independent of priming activity. We also directly measured the effect of TRF1 on primase activity, independent of DNA synthesis. Reaction mixtures included DNA pol α/primase, the single-stranded TS10 DNA substrate, and rNTPs and were incubated in the absence and presence of TRF1. The results demonstrated that TRF1 has no effect on the priming activity of DNA pol α/primase on telomeric DNA substrates (data not shown).

The concentration of TRF1 used in the primase-independent reactions is greater than those employed in the primase-dependent reactions, and the concentration of the DNA substrate was reduced. The amount of total DNA synthesis is considerably greater in the primase-independent reactions as they do not rely on the relatively inefficient primase activity. Thus, with a greater level of telomeric duplex DNA being generated, a larger amount of TRF1 was employed to ensure that all the duplex DNA being generated throughout the course of the reaction could be bound by TRF1. In addition, the concentration of the DNA substrate was reduced compared to that in the priming-dependent assays because

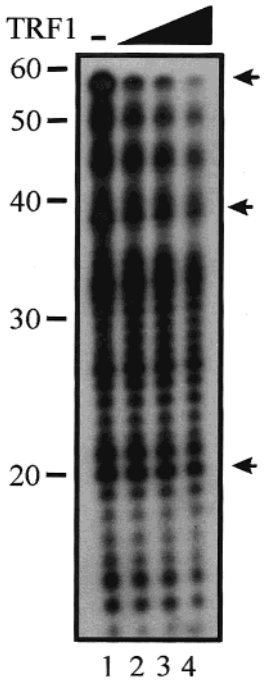

FIGURE 3: TRF1 inhibition of telomere C-strand priming and synthesis catalyzed by DNA pol α/primase. Telomere C-strand synthesis reactions were performed as described in Materials and Methods using the single-stranded TS10 substrate (5 pmol), and reaction mixtures were incubated at 37 °C for 30 min. An autoradiograph of C-strand synthesis reaction products performed in the absence of TRF1 (lane 1) or containing 50, 100, or 200 ng of TRF1 (lanes 2−4, respectively). The position of a 10 bp ladder is depicted on the left, and the specific products quantified by PhosphorImager analysis are denoted by the arrows on the right.

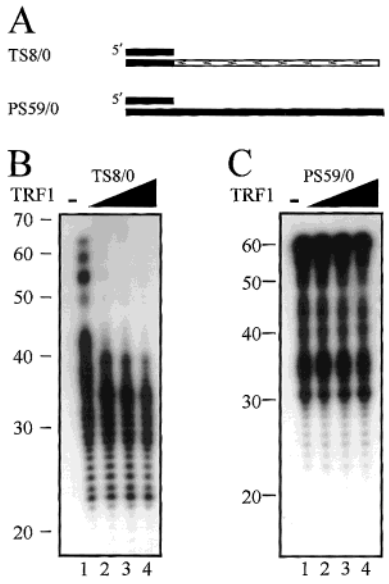

FIGURE 4: TRF1 inhibition is independent of primase activity and is specific for telomere DNA. Primase-independent C-strand synthesis reactions were performed with the partial duplex TS8/0 substrate (B) or the nontelomeric DNA substrate, PS59/0 (C). The structure for each substrate is depicted in panel A. Reactions with each substrate (0.25 pmol) were performed as described in Materials and Methods without TRF1 (lanes 1) or with 325, 750, or 1500 ng of TRF1 (lanes 2−4, respectively). Products were purified, separated, and detected by autoradiography.

of the efficient extension of the polymerase on these preprimed substrates. The data thus far support a mechanism whereby pol α, via copying the telomere G-strand, synthesizes TRF1 binding sites to which TRF1 binds and inhibits further pol α-catalyzed extension of the DNA substrate. The fact that the total amount of DNA synthesis was decreased in reactions performed with TRF1 suggests that pol α may be sequestered in a complex with TRF1 on the DNA. If pol α was released and free to extend other DNA substrates, the decrease in the amount of longer products would be accompanied by an increase in the amount of shorter products. If, however, the DNA substrate was limiting in the reactions, released pol α would not have any additional substrate to extend and also could account for the overall decrease in the extent of synthesis without an increase in the amount of shorter products. Importantly, reactions performed with increasing DNA substrate concentrations at constant TRF1 levels also did not reveal any increase in the amount of the shorter products. These data argue against TRF1-dependent release of pol α and support the sequestration of DNA pol α at the primer terminus (data not shown).

The model where TRF1 inhibits pol α synthesis of the telomere C-strand when bound to duplex telomere DNA would predict that inhibition by TRF1 would be specific for copying telomeric sequences capable of binding TRF1. A primer−template substrate which consisted of the same 18 bp duplex DNA sequence followed by single-stranded nontelomeric sequences with a similar GC content was prepared to test this hypothesis. Results obtained with this nontelomeric substrate, PS59/0, are shown in Figure 4C and demonstrate that TRF1 has no effect on DNA pol α-catalyzed synthesis of nontelomeric DNA. In addition, we observed no effect of TRF1 on DNA synthesis catalyzed by pol α using an activated calf thymus DNA substrate (data not shown). To rule out the possibility that telomeric DNA specific inhibition was the result of TRF1 binding to the single-stranded TS10 G-strand substrate, we performed DNA titration experiments. Increasing the DNA concentration using a fixed concentration of TRF1 revealed no effect on the level of synthesis observed by pol α (data not shown). If TRF1 was binding to the single-stranded DNA substrate, we would have expected to observe less inhibition as the DNA concentration was increased.

Results thus far have measured the effect of TRF1 on synthesis by pol α when TRF1 is bound upstream of the DNA polymerase. This is fundamentally different than the in vivo situation where TRF1 bound to duplex telomeric DNA is downstream of pol α. Therefore, a DNA substrate, termed DS/+4, was designed and constructed to determine the effect of TRF1 bound downstream of an advancing DNA polymerase. This substrate was designed to ensure the effect of TRF1 would be from the TRF1 bound downstream, and included the 18-bp duplex DNA followed by a single-stranded nontelomeric region of 41 bases. The nontelomeric DNA is essential to avoiding the synthesis of telomeric duplex DNA that could then be bound by TRF1 during the reactions. Following the nontelomeric single-stranded DNA were four duplex telomeric repeats. A 16 bp nontelomeric anchor sequence was employed to align the telomeric repeats in the proper register. Therefore, TRF1 could only be bound to the duplex telomeric DNA downstream of the advancing polymerase. We also prepared a control substrate, termed US/+4, to assess the degree of inhibition by TRF1 bound to four duplex telomeric repeats positioned upstream from the site of polymerase extension, again assessing the

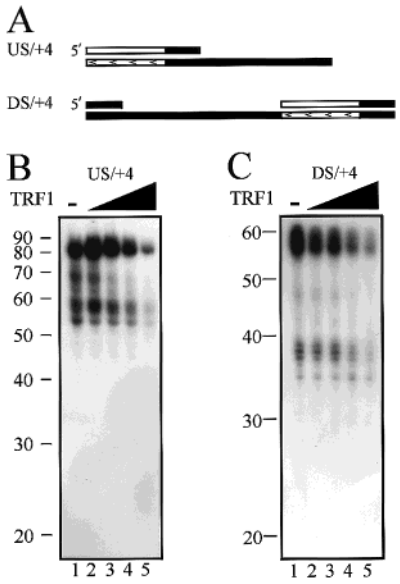

FIGURE 5: TRF1 inhibition is independent of position with respect to the direction of polymerase extension. Primase-independent synthetic reactions were performed using the US/+4 (B) or the DS/+4 substrate (C). The structure for each substrate is depicted in panel A. Reactions with each substrate (0.25 pmol) were performed without TRF1 (lane 1) or with 187, 375, 750, or 1500 ng of TRF1 (lanes 2−5, respectively). Products were purified, separated, and detected by autoradiography.

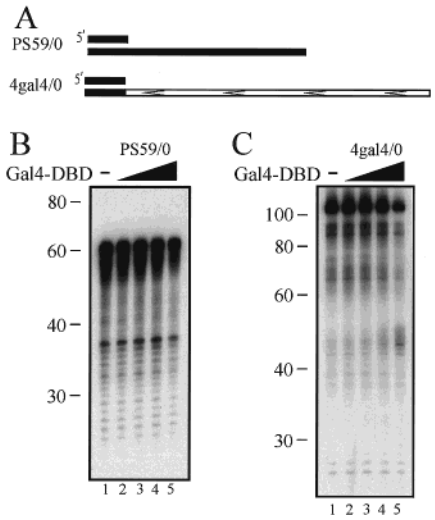

FIGURE 6: Gal4-DBD does not influence T7 polymerase catalysis. Primer extension reactions were performed using Sequenase (0.01 unit per assay) on the PS59/0 (B) and the 4gal4/0 substrate (C). The structure for each substrate is depicted in panel A. Reactions with each substrate (5 pmol) were performed without Gal4-DBD (lane 1) or with 125, 250, 500, or 1000 ng of Gal4-DBD (lanes 2−5, respectively). Products were purified, separated, and detected by autoradiography.

replication of nontelomeric DNA. The use of these two substrates allowed us to control for the affinity of TRF1 which varies with the number of telomere duplex repeats (*15*) and enabled the direct comparison of the TRF1 position with respect to the direction of polymerase extension. The results are presented in Figure 5 and demonstrate that from the upstream position, TRF1 exhibits a slight stimulation of synthesis followed by concentration-dependent inhibition (Figure 5B). At the highest level of TRF1, the extent of synthesis was reduced to 40% of that observed in the control reactions without TRF1. In contrast, from the downstream position (Figure 5C), the addition of TRF1 resulted in inhibition only, with a maximum level of inhibition of 85% at the highest TRF1 concentration. These results demonstrate that TRF1 inhibition of telomere C-strand synthesis can occur from both upstream and downstream positions with relation to the extending polymerase.

One possible mechanism of inhibition, as described above, is TRF1 blocking the polymerase from binding the substrate. A series of control assays were performed to determine the specificity of the inhibition. In these experiments, the DNA binding domain from the sequence specific double-stranded DNA binding protein Gal4-DBD was employed to bind tandem gal4 operators and determine the effect on the ability of the phage T7 polymerase (Sequenase) to copy the DNA template. As a further control, we assessed the effect of Gal4-DBD on Sequenase-catalyzed copying of the PS59/0 nontelomeric DNA, which also does not contain gal4 operator sequences. The results shown in Figure 6 reveal that Gal4-DBD has no effect on the level of synthesis until the highest concentration was reached. PhosphorImager analysis of the data revealed 10−15% inhibition at the highest concentration of Gal4-DBD. Using the Gal4 operator DNA substrate, again, no inhibition was observed until the highest concentration was used (Figure 6C). The degree of inhibition observed, as determined by PhosphorImager analysis, was similar to that observed for the non-gal4 operator containing DNA. The amount of Gal4-DBD used in the extension assays was based on an electrophoretic mobility shift assay of the purified protein with the duplex gal4 operator DNA such that the highest level was capable of binding 0.25 pmol of duplex DNA (data not shown). This level of binding is sufficient to bind all of the synthesized duplex gal4 operator DNA synthesized during the extension assay. These results suggest that the effect of TRF1 may be specific in that another double-stranded DNA binding protein could not elicit the same effect, specific inhibition of DNA synthesis.

A further control of the polymerase specificity was performed using the PS59/0 and TS8/0 DNA substrates assessing the effect of TRF1 on Sequenase-catalyzed extension. The level of TRF1 was chosen on the basis of mobility shift assays similar to those described for the Gal4-DBD to ensure the same level of DNA binding activity was used per assay. The results presented in Figure 7B demonstrate that TRF1 has no effect, as expected, on the ability of Sequenase to copy the nontelomeric PS59 substrate. Interestingly, TRF1 was able to significantly inhibit Sequenase-catalyzed copying of the telomeric DNA template in a concentration-dependent fashion (Figure 7C). Additional experiments performed under a variety of conditions reveal that the level of inhibition observed with Sequenase is similar to that observed for DNA pol α when the assays are performed under identical conditions. These results suggest that inhibition of synthesis is specific for TRF1, but not necessarily the polymerase used to copy the telomeric DNA sequence.

DISCUSSION

The regulation of telomere length in telomerase positive cells potentially involves the interplay of numerous protein factors present at the telomere. A recent study has suggested that the telomere can be present in an “open” or “closed”

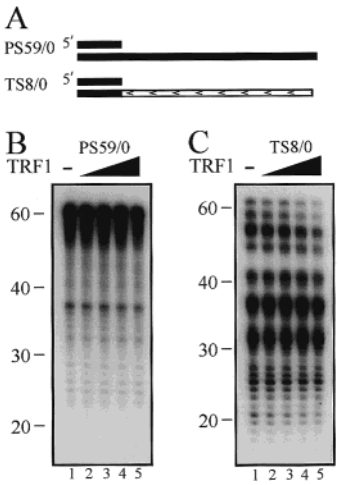

FIGURE 7: TRF1 inhibits T7 pol synthesis on telomeric DNA. Primer extension reactions were performed using Sequenase (0.01 unit per assay) on the PS59/0 (B) and the TS8/0 substrate (C). The structure for each substrate is depicted in panel A. Reactions with each substrate (5 pmol) were performed without TRF1 (lane 1) or with 187, 375, 750, or 1500 ng of TRF1 (lanes 2−5, respectively). Products were purified, separated, and detected by autoradiography.

state which dictates the accessibility of the telomere to enzymes required for lengthening or shortening of the telomeric DNA (*17*). The human proteins TRF1 and TRF2 have been demonstrated to be involved in the determination of the telomere state, open or closed. Interestingly, in an in vitro assay the addition of TRF1 to a duplex telomeric DNA substrate had no effect on the ability of telomerase to extend the telomeric G-strand (*17*). These results suggest that at least under the conditions employed and if the purified TRF proteins are capable of recapitulating the closed state in vitro, telomerase itself might not be the target of TRF1-dependent regulation. Our results demonstrate that purified TRF1 can both promote the closed state of a telomeric substrate and inhibits polymerase-catalyzed DNA synthesis on telomeric DNA sequences. All our assays have assessed the synthesis of the C-strand using a G-strand template. However, we would also be expected to observe TRF1 inhibition to occur if a DNA polymerase was synthesizing the telomere G-strand using a C-strand template, the consistent feature being TRF1 bound to duplex telomeric DNA creating a structure unable to support template-directed DNA synthesis by a DNA polymerase. A recent study employed the telomere C-strand synthesis assay and demonstrated that the hnRNP A1 protein was capable of inhibiting primase-dependent C-strand synthesis in vitro. The A1 protein and a truncated derivative UP1 are telomere specific single-stranded DNA binding proteins. The mechanism by which A1/UP1 inhibits primase-dependent C-strand synthesis is via blocking access of DNA pol α/primase to the single-stranded DNA substrate. The effect is apparently nonspecific as inhibition of C-strand synthesis was also observed when *E. coli* SSB and T4 single-stranded DNA binding protein gp32 were employed (*25*). Interestingly, A1/UP1 did not inhibit C-strand synthesis in reactions where a complementary DNA primer was added to the reaction mixtures. The conclusion was that A1/UP1 does not inhibit primase-independent C-strand synthesis, presumably as a result of an inability of A1/UP1 to bind the partially duplex DNA substrate (*25*).

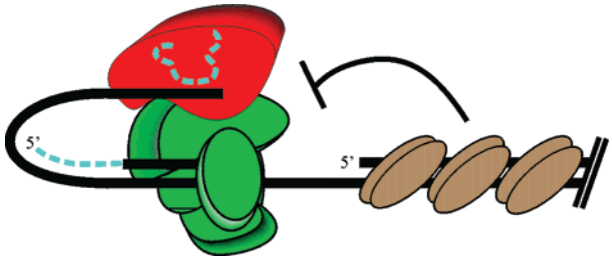

FIGURE 8: Model of TRF1 inhibition of telomere C-strand synthesis. TRF1 (brown) is depicted as a homodimer bound to duplex DNA downstream of the advancing telomere replication machinery. Telomerase (red) extending the G-strand is depicted in black with the RNA component denoted by the dashed blue line. DNA pol α/primase is depicted as a tetramer (green) extending an RNA primer (dashed blue line) in coordination with telomerase.

While it is clear that telomere length can be regulated at the level of duplex telomere DNA binding proteins, and the protein counting model has been observed in both yeast and mammals (*24*), the targets of these proteins remain largely undefined. A recent report using a yeast model system measuring the elongation of a shortened telomere has demonstrated that the rate of telomere elongation decreases as the telomere becomes longer (*22*). While the authors suggested that telomerase is the target of Rap1p action, the data presented cannot differentiate between telomerase-catalyzed G-strand synthesis or C-strand synthesis as being the target of Rap1p inhibition in yeast. If the likelihood that G- and C-strands are synthesized in a coordinated action is considered, originally demonstrated in *Oxytricha* (*26*), it is reasonable to hypothesize that these processes are coordinated in higher eukaryotes. TRF1 inhibition of telomere C-strand synthesis would result in the concomitant inhibition of G-strand extension by telomerase. The results would be consistent with the observation that the rate of telomere elongation decreases with increasing telomere length (*22*). A recent study has demonstrated that both primase and pol α are required for telomerase activity in vivo, further supporting a coordinated action C- and G-strand synthesis in vivo (*11*, *12*). A model for TRF1 inhibition of telomere C-strand synthesis is depicted in Figure 8 and is based on the protein counting model (*24*). We also have depicted telomere G-strand synthesis by telomerase and C-strand synthesis by pol α as occurring in a coordinated fashion (*26*). TRF1 bound to telomeric DNA can feedback from a downstream position to inhibit extension by DNA pol α (Figure 4B). While we have depicted pol α/primase in the model, and there is overwhelming in vitro and in vivo evidence demonstrating that this protein is involved in telomere C-strand synthesis, the possibility exists that polymerase δ may be responsible for C-strand synthesis following the RNA primer synthesis by pol α/primase (*9*−*12*).

The recent cytogenetic study of mammalian telomeres proposed a model whereby telomeres exist in a large loop encompassing the majority of the telomere (*27*). The terminal 3′ single-stranded G-strand overhang was proposed to exist in a classical D-loop annealing to the C-strand at a position near the nontelomere−telomere junction. A remaining question is what inhibits this 3′ OH from being extended by any of the eukaryotic polymerases present in the nucleus? One possibility is that TRF1, bound to duplex telomere DNA at positions upstream and downstream, blocks access to the

terminus or if a polymerase is bound inhibits extension of the 3′ OH. This is a slightly different scenario than that observed in lower eukaryotes where the single-stranded telomere DNA binding protein binds and restricts the access of telomerase to the 3′ OH by sequestering the terminus in an internal cleft between the α and β subunits of the protein (*28*, *29*). TRF1 does not directly restrict access to the 3′ OH being extended as demonstrated by inhibition when TRF1 was positioned downstream of the polymerase.

In addition to TRF1, TRF2 is localized to telomeric DNA (*15*), and recent evidence also has demonstrated that the Ku protein is associated with telomeres (*30*). One possibility is that in conjunction with the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), Ku could restrict access to the G-strand 3′ OH. A low-resolution structure of DNA-PKcs has suggested that single-stranded DNA termini could be sequestered within the protein subunit (*31*), reminiscent of the interaction of the *Oxytricha* telomere-binding protein (*29*). How TRF2, Ku, and potentially DNA-PK are assembled onto the telomeric DNA structures and their effect on maintenance of telomeres with respect to telomere replication remain to be determined.

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