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TRF1 Promotes Parallel Pairing of Telomeric Tracts *in Vitro*

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Human telomeres consist of long arrays of TTAGGG repeats bound to the telomere-specific proteins, TRF1 and TRF2. Here we describe the structure of *in vitro* complexes formed between telomeric DNA and TRF1 as deduced by electron microscopy. Visualization of TRF1 bound to DNA containing six or 12 tandem TTAGGG repeats revealed a population of DNAs containing a spherical protein complex localized just to the repeats. Mass analysis of the protein complexes suggested binding of TRF1 dimers and tetramers to the TTAGGG repeats. The DNA was not significantly compacted or extended by protein binding. TRF1 formed filamentous structures on longer telomeric repeat arrays ($\geqslant 27$ repeats) consistent with the presence of an array of bound TRF1 dimers. Unexpectedly, there was a strong propensity for two telomeric tracts to form paired synapses over the TRF1 covered segment. Up to 30% of the TRF1-bound DNAs could be found in a paired configuration with a strong bias for a parallel as contrasted to an antiparallel arrangement. TRF1-induced pairing was confirmed using a ligation assay which detected the formation of DNA multimers dependent on the presence of TRF1 and a 27mer repeat array in the DNA. These findings suggests that this protein may have an architectural role at telomeres. We discuss the possibility that TRF1-dependent changes in the conformation of telomeres are involved in the regulation of telomere length.

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Introduction

Mammalian telomeres contain two specific proteins in addition to nucleosomal chromatin (reviewed by Konig & Rhodes, 1997; Smith & de Lange, 1997). A candidate mammalian telomeric protein was initially identified biochemically as an activity that could bind to double-stranded TTAGGG repeats *in vitro*. The protein responsible for this activity, the TTAGGG repeat binding factor 1 (TRF1), was subsequently found associated with human and mouse telomeres throughout the cell cycle (Chong *et al.*, 1995). Mammalian telomeres were recently shown to contain a second telomeric protein, TRF2, which is a distantly related homologue of TRF1 with several distinctive features (Broccoli *et al.*, 1997b). Consistent with their presence along the length of the duplex TTAGGG repeat regions, both TRF1 and TRF2 bind *in vitro* to duplex TTAGGG repeats but not to single-

stranded telomeric DNA and the factors do not require a DNA end for binding (Zhong *et al.*, 1992; Broccoli *et al.*, 1997b). TRF1 and TRF2 have the same *in vitro* DNA binding specificity showing a strong bias for the vertebrate telomeric DNA sequence over similar repeats derived from the telomeres of other eukaryotes (Zhong *et al.*, 1992; Hanish *et al.*, 1994; Broccoli *et al.*, 1997b).

TRF1 carries a Myb-type helix-turn-helix motif at its C terminus (Chong *et al.*, 1995) and a novel dimerization domain for homotypic interactions in the middle of the polypeptide (Bianchi *et al.*, 1997). A third domain, recognized based on its conservation in the highly diverged human and mouse TRF1 proteins, is found at the N terminus and is composed of acidic residues (Chong *et al.*, 1995; Broccoli *et al.*, 1997a). Whereas TRF2 has the same general architecture as TRF1, its N terminus is significantly different, carrying mostly basic residues (Broccoli *et al.*, 1997b).

Which sequence within the telomeric TTAGGG repeat arrays is recognized by TRF1 is not known. Since TRF1 can bind to DNA as a homodimer, both Myb domains are likely to contact the same

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Abbreviations used: EM, electron microscopy; TRF1, telomere binding protein, 1.

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set of bases but neither this site nor the required distance between the Myb target sites has been established. TRF1 displays much stronger *in vitro* binding to DNA probes with six or 12 tandem TTAGGG repeats than to probes containing three repeats (Zhong *et al.*, 1992). The enhancement occurs in absence of cooperative interactions under conditions where each DNA probe binds a single TRF1 dimer. The enhanced binding to longer TTAGGG repeat arrays could be explained if the optimal site for TRF1 was larger that three repeats but other explanations have not been ruled out.

TRF1 plays an important role in the length regulation of human telomeres (van Steensel & de Lange, 1997). Human telomeres can be maintained by telomerase, a reverse transcriptase that uses an internal RNA to synthesize TTAGGG repeats onto chromosome ends (Morin, 1989, 1996; Feng *et al.*, 1995). Recent evidence has indicated that this process is regulated by a negative feedback mechanism that controls telomere length at a steady state setting (van Steensel & de Lange, 1997). The length setting of human telomeres can be disturbed by overexpression of TRF1 leading to a gradual shortening of the TTAGGG repeat tracts. Conversely, inhibition of TRF1 activity elongates the telomeres. The re-setting of telomere length by altered TRF1 levels is not accompanied by a change in telomerase expression, suggesting that TRF1 acts in *cis*, inhibiting telomerase at individual chromosome ends. Such a *cis*-acting regulatory mechanism explains how cells can monitor and modify the length of individual telomeres (Barnett *et al.*, 1993).

The current model for the action of TRF1 proposes that telomeres can switch between a state in which they are accessible to telomerase and a closed state in which the enzyme is blocked in *cis*. Binding of a critical mass of TRF1 to the telomeric TTAGGG repeat array is proposed to shift this equilibrium to the closed state. Such a telomere is expected to undergo progressive sequence loss with each cell division due to the enzymatic strategy of DNA replication and possibly also as a consequence of exonucleolytic degradation of the C-rich telomeric strand (Makarov *et al.*, 1997). The resulting shortening will eventually lower the number of bound TRF1 molecules allowing the telomere to switch to a state in which telomerase can elongate the chromosome end. This process can provide a dynamic feedback mechanism for telomere length control resulting in a stable length setting of telomeres as is observed in the germline and in tumor cell lines.

A similar mechanism for telomere length control operates to stabilize telomeres in the budding yeast, *Saccharomyces cerevisiae*. The length of yeast telomeres is regulated by the duplex telomeric DNA binding protein Rap1p (reviewed by Shore, 1994). While Rap1p is not a recognizable homolog of TRF1 or TRF2, these proteins are distantly related because each carries a Myb-type DNA binding domain. Recent data favor a protein-counting model for the telomere length homeostasis by

Rap1p (Marcand *et al.*, 1997). Tethering of Rap1p to a subtelomeric site resulted in a concomitant shortening of the telomere, consistent with the view that cells detect the overall mass of Rap1p present on each individual telomere.

How is the binding of a critical mass of telomeric protein along the duplex telomeric DNA translated into inhibition of telomerase in *cis*? One possibility is that binding of TRF1 alters the conformation of the telomere into a state that blocks telomerase from acting on the telomere terminus. As such an architectural role for TRF1 might be reflected in the structure of the TRF1-DNA complex formed *in vitro*, we initiated experiments to visualize and characterize TRF1-bound telomeric tracts using electron microscopy (EM). Here we report that TRF1 has the ability to change the configuration of telomeric DNA by inducing associations between arrays of TTAGGG repeats, forming parallel-paired synaptic structures. We suggest that this unusual feature of TRF1 contributes to its role in the regulation of telomere length.

Results

Morphology and size of TRF1 complexes bound to telomeric DNA sites

Purified baculovirus-produced $[His]_6$-tagged human TRF1 (Bianchi *et al.*, 1997) was allowed to bind to linear or circular plasmids carrying a stretch of 12 tandemly arranged full TTAGGG repeats and one half repeat ($(TTAGGG)_{12.5}$). The resulting complexes were fixed with glutaraldehyde and examined by EM. Based on previous gel-shift analysis, both circular and linear $(TTAGGG)_{12.5}$ plasmids are expected to bind to TRF1 efficiently (Zhong *et al.*, 1992). Examination of the complexes formed (Figure 1A) revealed fields of DNA molecules each with a single large protein ball associated with the DNA. In one experiment employing a ratio of approximately 30 TRF1 dimers per DNA molecule, 97% of the linear $(TTAGGG)_{12.5}$ plasmid contained a single protein complex roughly at the middle of the DNA (the repeat block lies between 35% and 38% from one end) and less than 5% of the DNA contained protein bound elsewhere or at the ends of the DNA. Measurements of the position of the protein complex from the nearest end of the $(TTAGGG)_{12.5}$ plasmid produced a sharp distribution between 35% and 40% with a mean of 37.6($\pm$3.6)% ($n = 102$) (Figure 1B). Similar results were obtained with a baculovirus-derived human TRF1 protein that lacked the N-terminal $[His]_6$ tag. These data show that TRF1 binds specifically to the TTAGGG repeat stretch in the plasmid.

We determined whether the DNA takes a relatively straight path through the TRF1 complex or whether it may wrap about the protein resulting in a significant foreshortening of the DNA. To examine this, a 326 bp DNA fragment carrying the $(TTAGGG)_{12.5}$ site slightly off center was com-
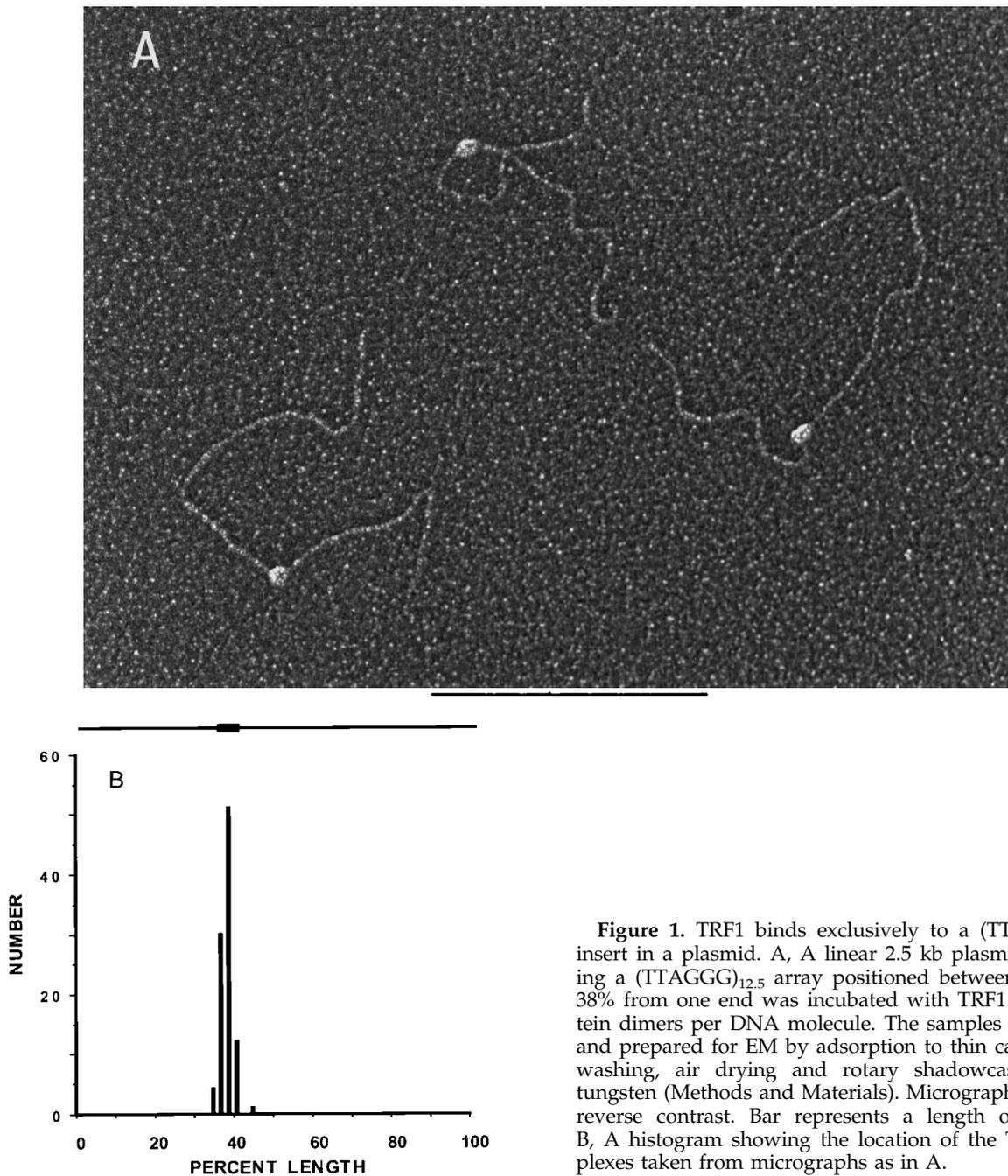
Figure 1. TRF1 binds exclusively to a $(TTAGGG)_{12.5}$ insert in a plasmid. A, A linear 2.5 kb plasmid containing a $(TTAGGG)_{12.5}$ array positioned between 35% and 38% from one end was incubated with TRF1 at 30 protein dimers per DNA molecule. The samples were fixed and prepared for EM by adsorption to thin carbon foils, washing, air drying and rotary shadowcasting with tungsten (Methods and Materials). Micrograph shown in reverse contrast. Bar represents a length of 1000 bp. B, A histogram showing the location of the TRF1 complexes taken from micrographs as in A.

plexed with TRF1 and examined (Figure 2A and B). Fields of the DNA fragment both bound by TRF1 and free of protein were photographed and the length of the DNA measured. The mean length of the protein-free DNA was 838($\pm$62) Å ($n = 30$) (shortened from the solution rise of DNA by the ethanol dehydration step). Measurement of the length of the DNA bound by TRF1 (taking the most direct path across the protein ball) yielded a mean value of 788($\pm$78) Å ($n = 49$), a value within 6% of the protein-free value. From this we conclude that the DNA is not wrapped about the protein ball nor is it extended in length due to TRF1 binding.

Based on two independent *in vitro* assays for DNA bending, Bianchi *et al.* (1997) concluded that the binding of one TRF1 dimer to telomeric DNA induces a shallow distortion (60° from a straight trajectory) in the site. Examination of TRF1 complexes at sites of 12.5 or 6.5 repeats did not reveal a distinct bend in the DNA, but a bend of this magnitude could have eluded detection, in particular if the distribution in bending angles was significant.

The mass of TRF1 bound to telomeric sites was determined by comparison to β-amylase particles (Griffith *et al.*, 1995; Allen *et al.*, 1997). Just prior to adsorption of the samples to the EM support,
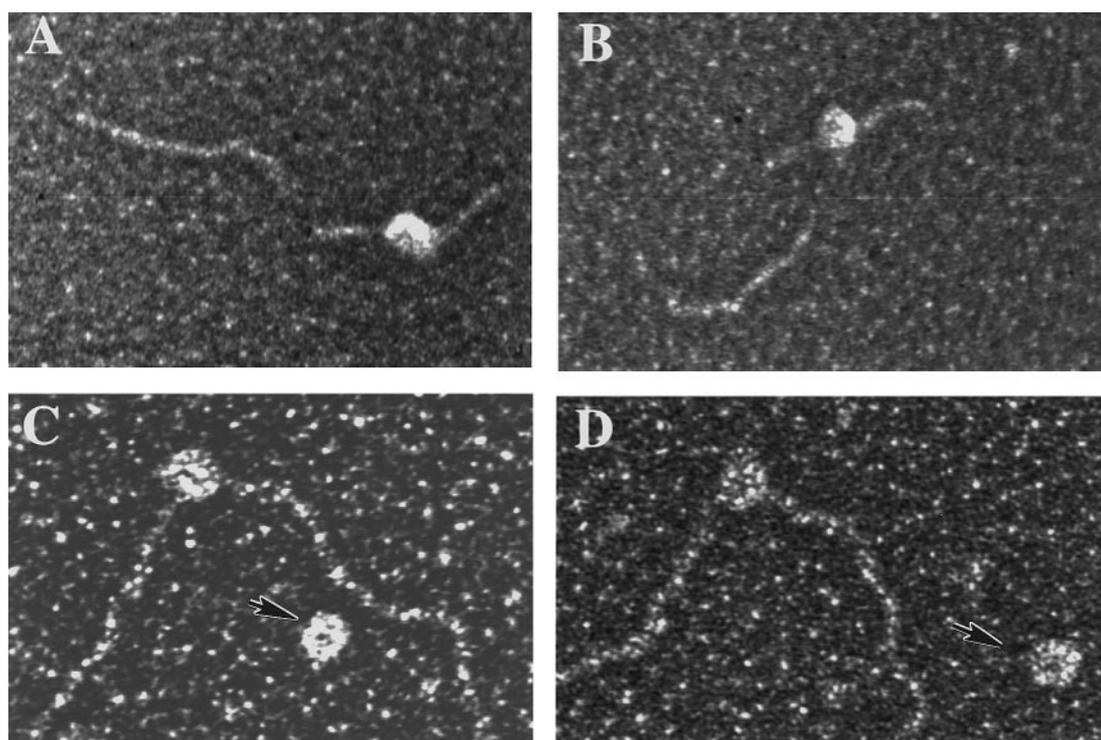
Figure 2. TRF1 binding to a $(TTAGGG)_{12.5}$ site appears as a discrete particle that does not shorten the DNA. A and B, TRF1 was incubated with a 326 bp DNA containing a central $(TTAGGG)_{12.5}$ array and prepared for EM as for Figure 1. Comparison of the protein-free and bound DNAs shows no significant shortening due to protein binding. C and D, Complexes of TRF1 bound to the $(TTAGGG)_{12.5}$ insert as for Figure 1 were formed, and just prior to preparation for EM were mixed with β-amylase molecules (200 kDa mass) (arrows) as a molecular mass marker. Bar represents a length of 500 bp.

β-amylase (200 kDa) treated with 0.6% (v/v) glutaraldehyde was added to the preparations resulting in images with numerous β-amylase molecules lying in close proximity to the TRF1-DNA complex (Figure 2C and D). Comparison of the mean size (projected area) of at least five β-amylase molecules for each TRF1 complex provides a means of correcting for local variations in metal coating and determining the mass of the TRF1-DNA complex. For this approach to provide a clear answer we needed only to distinguish between particles of ∼50, 100, 200 or 400 kDa (monomer, dimer, tetramer, octamers) since the monomer molecular mass is known. This same approach was used recently with the β-amylase marker in a study of the 100 kDa *Escherichia coli* MutS protein (Allen *et al.*, 1997) where it was shown that MutS binds at a G/T mismatch in DNA as a 200 kDa homodimer, and this was confirmed independently in solution by surface plasmon resonance.

Histograms representing the calculated mass derived from such measurements of the protein bound to the $(TTAGGG)_{12.5}$ site and to a shorter telomeric stretch (with six full repeats and one half repeat, $(TTAGGG)_{6.5}$) are given in Figure 3. While the exact nature of the TRF1 recognition site is not known, it was previously shown that TTAGGG arrays of six repeats or longer encompass the optimal binding site for

TRF1 (Zhong *et al.*, 1992). The mass of $[His]_6$-tagged hTRF1 is 54 kDa and the mass of each TTAGGG repeat is ∼4 kDa. If we assume that the DNA takes a straight path through the complex and adds to the volume in a way roughly equivalent to the protein, it is reasonable to subtract the mass of the DNA to estimate the protein mass. For the shorter $(TTAGGG)_{6.5}$ site (Figure 3A) the mean value of the complex was 236($\pm$28) kDa ($n = 49$). Subtracting 25.7 kDa for 6.5 telomeric repeats results in a mass of 214($\pm$26) kDa, equivalent to four times 54 kDa. This suggests that the most common binding form was that of a particle containing a TRF1 tetramer but with a distribution (Figure 3A), indicating that the $(TTAGGG)_{6.5}$ site can accommodate up to three dimers. The distribution on the $(TTAGGG)_{12.5}$ site was also consistent with the interpretation of most DNAs being bound by one tetramer and occasional acquisition of additional dimers or tetramers (Figure 3B).

To verify that in agreement with the finding of Bianchi *et al.* (1997), a similar EM analysis of TRF1 in the absence of DNA would detect dimer sized particles β-amylase and TRF1 were each diluted and mounted on separate EM supports (if mixed the two could not be distinguished one from the other) and shadowcast in parallel. Analysis of the
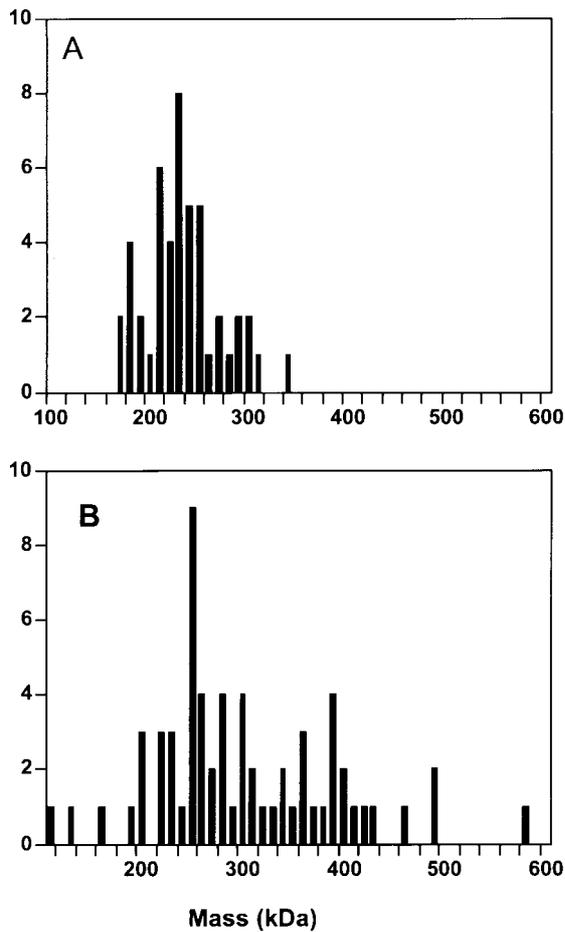
Figure 3. Mass analysis of TRF1 complexes formed over TTAGGG repeats. Complexes of TRF1 were formed on linear plasmids containing inserts of $(TTAGGG)_{6.5}$ (A) or $(TTAGGG)_{12.5}$ (B) together with β-amylase as an internal marker as shown in Figure 2. Mass for each TRF1 complex was determined as described in Materials and Methods.

projected particle area for 65 particles each yielded a calculated mass of the TRF1 particles of $84(\pm28)$ kDa, consistent with a majority of particles as dimers but with some monomers present, likely due to the high dilution employed prior to fixation. For both DNAs the protein complexes appeared round with little indication of their being formed from two or more separate protein dimers. This may in part reflect some compaction of the protein by the fixation process. The EM studies were purposely carried out using levels of TRF1 (one or more TRF1 monomers per repeat) above which no further increase in protein binding appeared to occur, as seen by EM. The close packing of two dimers on the tract with six repeats resulted in a peak in the histogram of apparent tetramer size. This, however, should not be taken as suggesting that the binding form of TRF1 consists of tetramers rather than dimers.

TRF1 pairs long telomeric tracts into synaptic structures

To analyze the interaction of TRF1 with the long telomeric tracts more representative of actual telomeric DNA, we employed two plasmids carrying extensive tracts of TTAGGG repeats. One plasmid (pTH5) carried a stretch of 27 precise tandem TTAGGG repeats, referred to as $(TTAGGG)_{27}$. The second plasmid (pSP73.Sty11) contained an 800 bp segment made up of arrays of TTAGGG repeats and TTGGGG repeats (see Materials and Methods for details), referred to as $(TTRGGG)_{130}$. TTGGGG repeats constitute weaker but significant TRF1 binding sites (L. Chong & T. de L., unpublished observations).

When the $(TTRGGG)_{130}$ site was incubated with low amounts of TRF1 (ratio less than 0.5 TRF1 monomers per repeat), the protein formed spherical balls (size, one to two dimers) distributed along the repeat tracts in a manner indicative of little or no cooperativity (Figure 4A, and data not shown). Lack of strong cooperativity for TRF1 binding was also documented by Bianchi *et al.* (1997). However, when higher amounts of TRF1 were used the complete $(TTRGGG)_{130}$ tract could be covered with protein (Figure 4B). Similarly, single protein balls or extensively covered tracts could be observed on the $(TTAGGG)_{27}$ site depending on the protein concentration (not shown).

The mean length of the TRF1 tracts on the $(TTAGGG)_{27}$ sites was 412 Å ($n = 28$) ranging from 350 to 510 Å. Given that a 164 bp (27×6) DNA fragment prepared by EM by these methods will measure ~490 Å, this is consistent with relatively little shortening of the DNA in these longer TRF1 covered tracts. Similarly, the length of TRF1-covered $(TTRGGG)_{130}$ site appeared close to the length of the protein-free repeat array (Figure 4B). When varying amounts of TRF1 was added to a covalently closed plasmid containing the $(TTRGGG)_{130}$ site, the protein-complexed plasmid remained coiled on itself (Figure 4C), suggesting that TRF1 does not significantly unwind the DNA upon binding.

Examination of fields of complexes formed by the binding of a moderate excess of TRF1 (ratios of two protein monomers per repeat) to the $(TTAGGG)_{27}$ and $(TTRGGG)_{130}$ sites revealed a strong tendency for the DNAs to associate with each other over the region covered by TRF1 (Figure 5). This effect was not due to the $[His]_6$ tag on TRF1, since pairing was also observed with the untagged protein (data not shown). While this behavior was never observed with DNAs containing less than 12 repeats, and only seen occasionally with the $(TTAGGG)_{12.5}$ site, it became pronounced with repeats tracts of $n = 27$ or more. When TRF1 was added at ratios of five or more monomers per telomeric repeat, much of the DNA was sequestered in aggregates containing many DNA molecules. With TRF1 in the range of two monomers per repeat, aggregates were less common and the
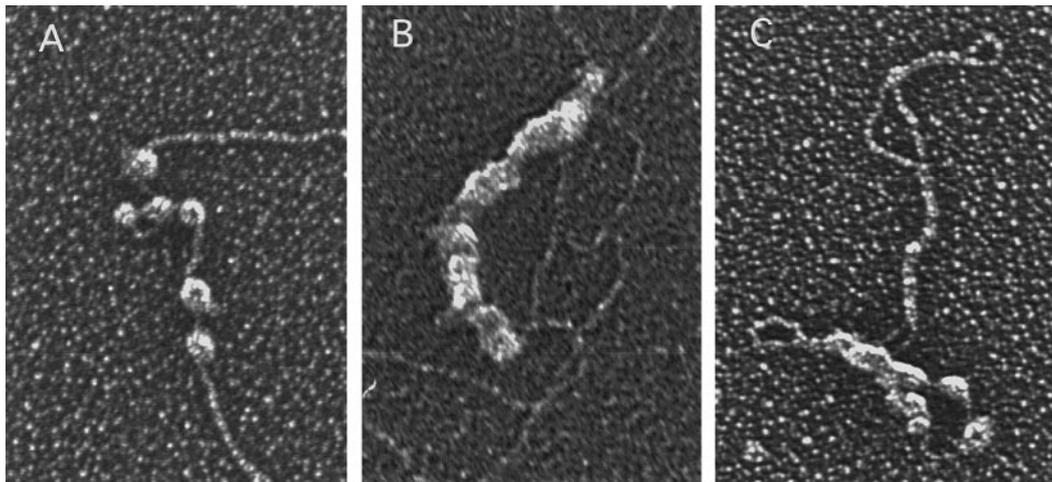
Figure 4. TRF1 binding to long telomere repeats is non-cooperative, does not unwind DNA and may exhibit a regular substructure. TRF1 complexes were formed on linear (A and B) or supertwisted (C) plasmids containing a $(TTRGGG)_{130}$ repeat at subsaturating (A) or saturating (B and C) TRF1 concentration. The supercoiled DNA in C shows no significant unwinding due to protein binding. Bar represents a length of 1000 bp.

$(TTAGGG)_{27}$ and $(TTRGGG)_{130}$ sites were frequently arranged into synaptic structures containing two DNAs paired over the protein-covered segments (Figure 5A to D). Synaptic structures were equally common with circular and linear DNA, and with linear DNA carrying the $(TTRGGG)_{130}$ stretch at one end (representing the natural orientation of telomeric DNA with the G-rich strand extending to the 3′ end). Synapses involving three DNAs were extremely rare.

Under optimal conditions for synapsis of the $(TTAGGG)_{27}$ site by TRF1 (Materials and Methods)
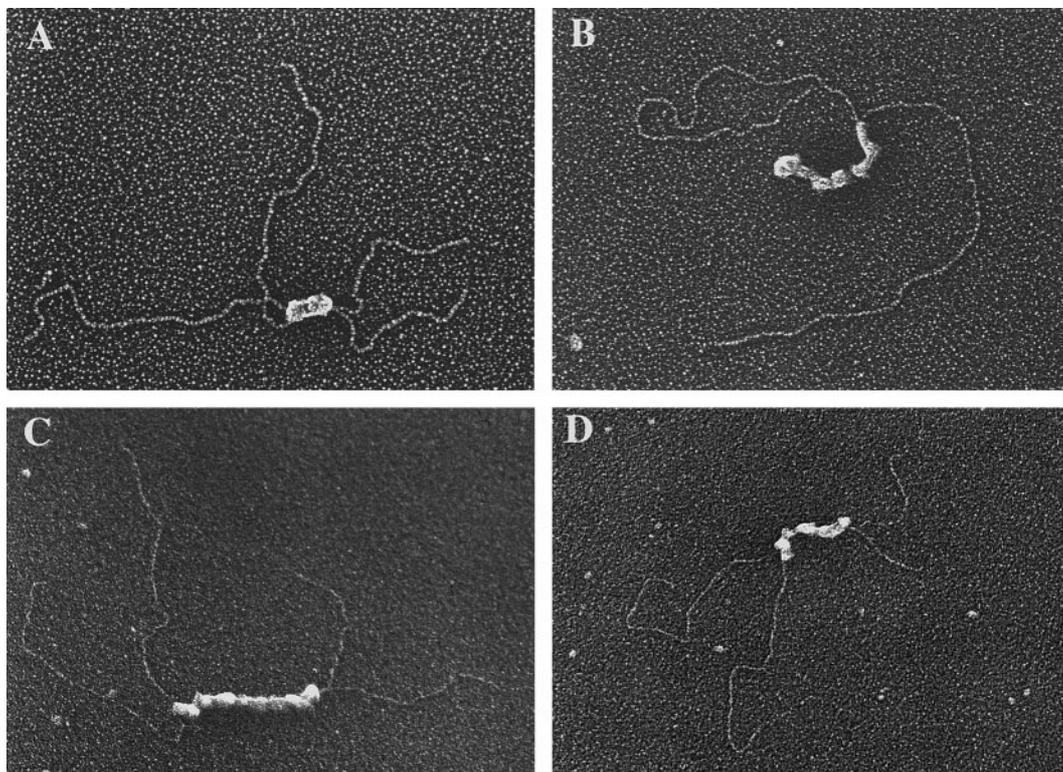

Figure 5. TRF1 binding to long TTAGGG repeat arrays promotes parallel pairing. TRF1 complexes were formed on linear ~3 kb plasmid DNAs containing a single $(TTAGGG)_{27}$ insert (A) or a $(TTRGGG)_{130}$ insert (B to D) located at the end of the DNA (B) or off set about one-third the distance from one end (A, C and D) and prepared for EM. Bar represents a length of 1000 bp (A, B and C) and 1400 bp (D). Micrographs in C and D were taken at a 40° tilt in the EM to enhance contrast and all are shown in reverse contrast.

greater than 99% ($n = 361$) of the DNAs were covered by TRF1 at the site of the repeats and 27% (98) were present in synaptic pairs. Because the repeat in the linearized (TTAGGG)$_{27}$ plasmid is asymmetrically positioned, it was possible to visually determine whether the DNAs in the synaptic pairs were arranged parallel or antiparallel with respect to each other. Here 27 of the 49 were ones in which the DNA arms were clearly laid out as in Figure 5 and for these structures there was a 13:1 preference for parallel over antiparallel arrangement of the DNA. A strong preference for parallel arrangement was also observed for TRF1 pairing of linear (TTRGGG)$_{130}$ plasmid and for the latter DNA in one experiment there was a 4:1 ratio of parallel to antiparallel configurations.

The observation that the diameter of the TRF1 tract in the region of synapses was visually greater than that of a single TRF1 tract on unpaired DNA suggested that pairing involved two TRF1 covered tracts aligning with each other as contrasted to a single protein tract encompassing two DNAs over the region of the TTAGGG repeats. To determine whether the DNAs might be interwound or partially base-paired within the region of the synapse, synaptic structures were generated on linear (TTAGGG)$_{27}$ DNA followed by incubation with the psoralen derivative HMT and then exposed to UV light (Makhov *et al.*, 1996). If the 4 DNA strands in the synapse were intimately associated with each other then some cross-linking of the DNA duplexes by HMT should occur (Makhov *et al.*, 1996). Following deproteinization and examination of the DNA by surface spreading EM, no paired DNAs were observed ($n = 100$; data not shown) arguing against close association of the four DNA strands in the synapse.

TRF1-induced pairing monitored by a ligation assay

To further verify that the pairing of telomeric DNA by TRF1 was not due to some aspect of the EM techniques employed, we examined the effect of TRF1 in a ligation assay. Reasoning that TRF1-mediated pairing of TTAGGG repeat sites would increase the rate of intermolecular ligation of the TRF1-bound fragments, we tested whether the formation of ligated dimers was influenced by binding of the protein. An end-labeled 217 bp Asp718 fragment containing 27 TTAGGG repeats was incubated with increasing amounts of TRF1 and a fixed amount of DNA ligase. The reaction products were deproteinized and analyzed by native acrylamide gel electrophoresis. The results indicated that TRF1 enhanced the joining of two TTAGGG repeat fragments into a 434 bp dimer by DNA ligase (Figure 6). Due to the low DNA and ligase concentrations in these experiments, no ligation of dimers occurs in the absence of TRF1. At high concentrations of TRF1, circularization of the DNA occurred, consistent with the propensity of TRF1 to induce a shallow bend in its substrate (Bianchi
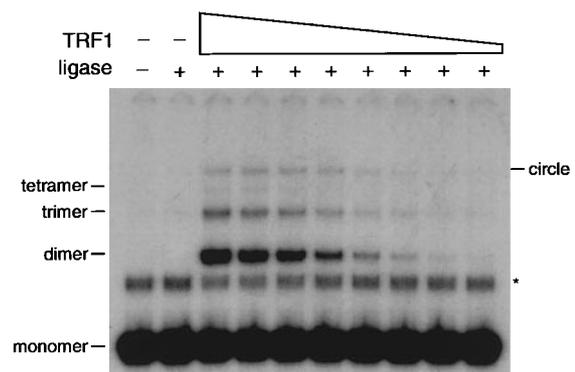
Figure 6. TRF1-mediated synapsis assayed by ligation of substrate dimers. A (TTAGGG)$_{27}$ fragment was incubated with DNA ligase in the presence of decreasing amounts of TRF1 (ranging from 1920 to 15 ng/ml in twofold dilution steps in consecutive lanes) as indicated above the lanes. The positions of the monomeric (217 bp) fragment as well as the dimeric, trimeric, tetrameric, and circular ligation products are indicated.

et al., 1997). In addition, trimers and tetramers were formed at high TRF1 concentration. Enhanced dimer ligation did not occur with heat-inactivated TRF1 nor when a control fragment lacking a TRF1 binding site was incubated with the protein (data not shown). These results argue that the TRF1-induced pairing observed in EM also occurred under other experimental conditions.

Discussion

TRF1 is a duplex telomeric DNA binding protein that regulates telomere maintenance in human cells. Here we describe the ultrastructure of the DNA-protein complexes formed by telomeric tracts bound to TRF1 *in vitro*. TRF1 formed long protein filaments along telomeric repeat arrays and displayed a strong tendency to induce parallel pairing of such tracts. This unusual feature of TRF1 may reflect how TRF1 contributes to telomere length homeostasis and other telomeric functions *in vivo*.

TRF1 was found to form three distinct classes of complexes with telomeric DNA *in vitro*. When binding to short telomeric tracts composed of six or 12 telomeric repeats, TRF1 formed a single round particle consisting of two protein dimers. The DNA in this and other complexes was not wrapped around TRF1 nor did we detect distortions in the telomeric DNA tracts. A second type of complex was formed when TRF1 was allowed to interact with long arrays of telomeric DNA. On such substrates, TRF1 formed a densely packed protein filament that appeared to cover the complete telomeric repeat array. The formation of these complexes did not involve strong cooperative interactions between individual TRF1 binding units, since at low protein concentrations the long telomeric tracts often contained several isolated protein balls that did not show obvious clustering.

Lack of strong cooperativity is consistent with results obtained in gel-shift assays (Bianchi *et al.*, 1997). Filaments of TRF1-coated telomeric tracts were not perceptibly curved or twisted.

The third type of TRF1 complex revealed the formation of striking higher-order structures of likely biological significance. When TRF1 was allowed to bind to long telomeric tracts at levels that nearly fully saturated the binding sites, a considerable fraction of the complexes were composed of two DNA molecules held together by a filament(s) of TRF1 protein. The resulting structures resembled a synaptic complex in which the telomeric tracts were positioned in a parallel conformation. Parallel pairing of telomeric tracts occurred with linear as well as circular DNA and was efficient with DNAs in which the TTAGGG repeat array extended to the 3′ end of the molecule, an arrangement representing the natural configuration of telomeric repeat array at chromosome ends. The specificity and frequency with which TRF1 induced telomere synapsis as well as the fact that TRF1 promoted intermolecular ligation of TTAGGG repeat-containing DNA molecules in solution strongly suggests that the EM analysis reveals a relevant activity of this protein. While the pairing observed here formally fits the working definition of a paranemic joint used in studies of homologous pairing proteins such as RecA protein (reviewed by Griffith & Harris, 1988), here it is likely that pairing is mediated by protein-protein interactions as contrasted to DNA-DNA or DNA-protein interactions as in the case of RecA.

Studies of the interaction of TRF1 with TTAGGG repeat tracts of varying length could provide an invaluable paradigm for understanding the behavior of other proteins that interact with microsatellites, either in the form of duplex DNA or RNA. Of particular interest are the triplet repeats whose expansion in the human genome results in myotonic dystrophy $((CTG)_n)$ and the Fragile X syndrome $((CCG_n))$ (Wells, 1996). In these diseases the biological consequences of the repeat tracts are observed only after the repeat tracts have grown beyond a certain size threshold. Understanding of the nature of such thresholds may require elucidation of how the presentation of longer and longer arrays of binding sites to a DNA or RNA binding protein can generate higher-order structures with much greater stabilities or the capacity to generate intermolecular interactions.

This EM analysis revealed the ability of TRF1 to induce conformational alterations in the arrangement of telomeric DNA. With the relatively short (<1kb) telomeric tracts used in these studies TRF1 primarily induced intermolecular pairing of telomeric DNAs. However, we expect that given a longer stretch of telomeric DNA and sufficient numbers of bound TRF1 molecules, telomeric pairing could be induced intramolecularly resulting in a coiled structure. We propose that this conformational alteration occurs *in vivo* when long telomeres recruit a large number of TRF1 molecules.

The switch into the coiled structure induced by TRF1 could render the telomere inaccessible to telomerase, resulting in TRF1 mediated telomere length control. Whereas coiled telomeres have been occasionally noted in EM studies of interphase and metaphase chromosomes (Steinmüller *et al.*, 1993; Ludérus *et al.*, 1996), it will be a challenge to determine the relevance of these structures to TRF1-mediated telomere length control.

Telomere associations have been suggested to play a role in chromosome synapsing in prophase of meiosis I (reviewed by (Dernburg *et al.*, 1995), in an anaphase checkpoint in mitosis (Cenci *et al.*, 1997; Kirk *et al.*, 1997), in a second pathway for telomere size control uncovered in yeast (Li & Lustig, 1996), and in genome instability in malignant human cells (reviewed by de Lange, 1995). Obviously, the ability of TRF1 to mediate pairing of telomeric repeat tracts could play a key role in these aspects of telomere biology.

Materials and Methods

DNA substrates and proteins

Purified baculovirus-produced $[His]_6$-tagged human TRF1 was purified as described (Bianchi *et al.*, 1997) and stored at a concentration of 1 mg/ml in a buffer containing 500 mM KCl, 20% (v/v) glycerol, 3 mM $MgCl_2$, 0.1% (v/v) NP40, 20 mM Hepes (pH 7.9), 0.5 mM EDTA at −80°C. Human TRF1 lacking the $[His]_6$ tag was purified from baculovirus by $(NH_4)_2SO_4$ precipitation and chromatography over phosphocellulose as described (Chong *et al.*, 1995).

The plasmids used in this study contained 6.5, 12.5, and 27 perfect TTAGGG repeats (termed pTH6, pTH12, and pTH5, respectively) that had been cloned into the multiple cloning site of the 2464 bp plasmid pSP73 (de Lange *et al.*, 1990; Zhong *et al.*, 1992). The plasmids were prepared by standard methods and cleaved with *Pvu*I to place the repeats roughly one-third the distance from one end. The plasmid pSP73.Sty11 containing 130 TTRGGG repeats is described in the text and was cleaved with *Pvu*I to place the repeat block one-third the distance from one end, or with *Cla*I to place the repeat block at the end of the DNA. The 326 bp fragment containing 12.5 repeats was prepared by cleavage of pTH12 with *Nde*I and *Hpa*I.

Preparation of protein-DNA complexes and EM

The buffer used to prepare protein-DNA complexes consisted of 20 mM Hepes 0.150 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA. Reactions included DNA at 1 to 10 µg/ml and TRF1 at 0.5 to 5 protein monomers per TTAGGG repeat, as indicated in the text. Incubation was for 30 minutes, either on ice or at room temperature.

For preparation of crosslinked protein-DNA complexes, reactions were terminated by addition of glutaraldehyde to 0.6%. After incubation for five minutes at room temperature, the samples were filtered through columns of Bio-Gel A-5 m (BioRad Inc.), equilibrated with 0.01 M Tris-HCl (pH 7.6), 0.1 mM EDTA. Filtered samples were mixed with a buffer containing spermidine, adsorbed to glow-charged thin carbon foils, dehydrated through a water-ethanol series and rotary

shadowcast with tungsten as described (Griffith & Christiansen, 1978). Samples were visualized in a Philips CM12 instrument. Micrographs for publication were scanned from negatives using a Nikon multiformat film scanner and the contrast optimized and panels arranged using Adobe Photoshop. Morphometry measurements were done using a Summagraphics digitizer coupled to a Macintosh computer programmed with software developed by J.D.G.

EM mass measurement of the TRF1 particles bound to DNA followed the method described by Griffith *et al.* (1995). In brief, TRF1-DNA complexes were fixed with 0.6% (v/v) glutaraldehyde for five minutes on ice and isolated by gel filtration. Just prior to adsorption to the EM supports, β-amylase (Sigma), which had been fixed in the same manner, was added to a concentration of 1 μg/ml and the mixture processed for EM. Micrographs were taken of fields in which a TRF1 complex was present on the DNA, and there were at least five β-amylase particles nearby. Using a COHU CCD camera attached to a Macintosh computer and the NIH IMAGE software, the mean projected area of the β-amylase particles was determined and compared to the projected area of the TRF1 particle. Calculation of the TRF1 particle mass based on the 200 kDa mass of β-amylase was as described (Griffith *et al.*, 1995).

Ligation assay for synapse formation

A kinase end-labeled 217 bp Asp718 fragment containing an array of 27 TTAGGG repeats was used as a TRF1 binding substrate (Bianchi *et al.*, 1997). The DNA was incubated for 20 minutes at room temperature with baculovirus-derived TRF1 in 20 mM Hepes-KOH (pH 7.9), 200 mM KCl, 10 mM $MgCl_2$, 2 mM DTT. Reactions were carried out with 35 ng/ml of DNA and TRF1 protein concentrations ranging between 15 and 1920 ng/ml. ATP was added to 1 mM and T4 DNA ligase to 10 units/ml. Ligation reactions were performed at 23°C for 30 minutes and stopped by the addition of 0.5 volume of stop buffer (75 mM EDTA, 3 mg Proteinase K/ml, 15% glycerol) followed by incubation at 55°C for 15 minutes. Samples were size-fractionated on a 6% (w/v) polyacrylamide gel (29:1 ratio of acrylamide bisacrylamide) in 1 × TBE (8.9 mM Tris base, 8.9 mM boric acid, 2 mM EDTA, pH 8.3).

Acknowledgments

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