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TRF1 is a dimer and bends telomeric DNA

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TRF1 is a mammalian telomeric protein that binds to the duplex array of TTAGGG repeats at chromosome ends. TRF1 has homology to the DNA-binding domain of the Myb family of transcription factors but, unlike most Myb-related proteins, TRF1 carries one rather than multiple Myb-type DNA-binding motifs. Here we show that TRF1 binds DNA as a dimer using a large conserved domain near the N-terminus of the protein for TRF1–TRF1 interactions. Dimerization was observed both in a complex with DNA and in the yeast two-hybrid assay. TRF1 dimers were found to require both Myb repeats for the formation of a stable complex with DNA, indicating a parallel between the DNA-binding mode of TRF1 and other Myb-related proteins. TRF1 was found to have a number of biochemical similarities to Rap1p, a distantly related DNA-binding protein that functions at telomeres in yeast. Rap1p and TRF1 both require two Myb motifs for DNA binding and both factors bind along their cognate telomeric sequences without showing strong cooperative interactions between adjacent proteins. Furthermore, TRF1 was found to bend its telomeric site to an angle of ~120°. Since Rap1p similarly distorts telomeric DNA, we propose that DNA bending is important for the function of telomeres in yeast and mammals.

Keywords: DNA bending/Myb/Rap1p/telomeric protein/TRF1

Introduction

Telomeres, the specialized nucleoprotein complexes at the ends of eukaryotic chromosomes, have several functions (reviewed in Zakian, 1995b). They circumvent the end-replication problem of linear DNA molecules and protect DNA ends from degradation and fusion. In *Tetrahymena*, impaired telomere function leads to a defect in cytokinesis and to cell death (Yu *et al.*, 1990). Similarly, in yeast, loss of a single telomere results in cell cycle arrest and chromosome instability (Sandell and Zakian, 1993), and cells undergoing generalized telomere shortening eventually senesce (Lundblad and Szostak, 1989; Singer and Gottschling, 1994). Finally, in human cells, telomere length changes have been implicated in the molecular clock controlling cell senescence (Counter *et al.*, 1992; reviewed in Harley, 1995) and as a step in tumorigenesis (Counter *et al.*, 1994; reviewed in de Lange, 1995; Autexier and Greider, 1996).

Human telomeres are composed of long tandem arrays of the telomeric repeat TTAGGG, running with 5′ to 3′ polarity towards the end of the chromosome (Moyzis *et al.*, 1988; de Lange *et al.*, 1990). This sequence is maintained by telomerase, a ribonucleoprotein that uses an internal RNA template to synthesize tandem arrays of telomeric repeats onto chromosome ends (Morin, 1989; Blackburn, 1993). The TTAGGG repeat arrays are the only DNA requirement for telomere function in somatic human cells (Wilkie *et al.*, 1990; Farr *et al.*, 1991; Barnett *et al.*, 1993; Hanish *et al.*, 1994).

The only known protein components of mammalian telomeres are the TRF proteins, duplex TTAGGG repeat-binding factors that are localized at telomeres in interphase and metaphase chromosomes (Zhong *et al.*, 1992; Chong *et al.*, 1995; Ludérus *et al.*, 1996; Broccoli *et al.*, 1997; for review, see Smith and de Lange, 1997). Human TRF1 (hTRF1) is a low abundance activity found in nuclear extracts from all human cells and tissues, and a similar activity is present in other vertebrates (Zhong *et al.*, 1992; Chong *et al.*, 1995). TRF2 (also referred to as orf2) was identified recently as a TRF1 homolog that also localizes to telomeres (Bilaud *et al.*, 1996; D.Broccoli, A.Smogorzewska, L.Chong and T.de Lange, in preparation). TRF1 was recently shown to be involved in the regulation of telomere length in human cells (van Steensel and de Lange, 1997). TRF1 behaves as a negative regulator of telomere maintenance, probably by inhibiting the activity of telomerase at the ends of individual telomeres. Similarly, duplex telomeric DNA-binding activities in yeast have been implicated in telomere length control (reviewed in Shore, 1994; Zakian, 1995a; see also McEachern and Blackburn, 1995; Krauskopf and Blackburn, 1996).

TRF1 has DNA-binding properties *in vitro* that are consistent with its presence along the double-stranded telomeric repeat array at chromosome ends. TRF1 binds efficiently to arrays of duplex TTAGGG repeats, irrespective of the presence of a DNA terminus (Zhong *et al.*, 1992). Single-stranded telomeric DNA is not an effective TRF1 substrate and neither are heterologous telomeric sequences, such as double-stranded arrays of TTGGGG, TTAGGC, TTTAGGG, TTAGGGGG and TAGGG repeats (Zhong *et al.*, 1992; Hanish *et al.*, 1994; Chong *et al.*, 1995). This sequence specificity of TRF1 matches the sequence requirements for *de novo* telomere formation in human cells, suggesting that the TRF proteins are involved in this process (Hanish *et al.*, 1994).

Interestingly, TRF1 binding is stimulated by longer repeat arrays, with six or 12 repeats providing a better binding substrate than three repeats (Zhong *et al.*, 1992). Since DNA fragments with three, six or 12 telomeric

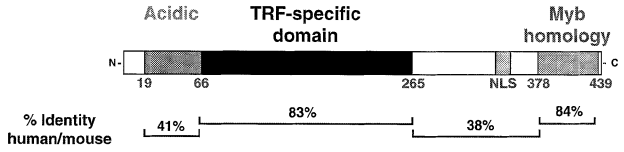

Fig. 1. Schematic of the domains of human TRF1 (hTRF1) and their conservation in mouse TRF1 (mTRF1) (adapted from Broccoli *et al.*, 1997).

repeats each bind exactly the same protein mass, this enhancement is not due to cooperative interactions between multiple TRF1-binding units. The minimal TRF1-binding site and the mechanism by which this protein differentiates between telomeric arrays of different lengths remain to be determined.

Mouse and human TRF1 are novel proteins with three recognizable domains: an acidic domain at the N-terminus, a conserved TRF-specific domain and a C-terminal domain with strong homology to the DNA-binding domain of Myb oncoproteins (see Figure 1; Chong *et al.*, 1995; Broccoli *et al.*, 1997). The c-Myb oncoproteins are transcriptional activators that carry three imperfect 50 amino acid repeats, two of which are required for DNA binding. In c-Myb, the two Myb repeats fold into helix–turn–helix (HTH) motifs that are closely packed on the DNA such that their recognition helices together contact a single short PyAACNG site (Ogata *et al.*, 1994). In other Myb-related DNA-binding proteins, Myb repeats have been found in four configurations: three tandem repeats (e.g. in the yeast protein BAS1, Hovring *et al.*, 1994), two tandem repeats (in many plant transcription factors, Ramachandran *et al.*, 1994; and in the fission yeast protein cdc5, Ohi *et al.*, 1994), two repeats separated by a linker (in the yeast proteins Reb1p and Rap1p and in the mouse protein MIDA1, Morrow *et al.*, 1993; Konig *et al.*, 1996; Sitzmann *et al.*, 1996) and single Myb repeats (in several yeast, plant, *Drosophila* and mouse proteins, England *et al.*, 1991; Brigati *et al.*, 1993; da Costa e Silva *et al.*, 1993; Baranowskij *et al.*, 1994; Lugert and Werr, 1994; Stokes and Perry, 1995). The group of proteins with one Myb repeat, which includes TRF1 and TRF2, had presented a conundrum, since in other Myb-related factors at least two Myb repeats are required for DNA binding (Henry *et al.*, 1990; Saikumar *et al.*, 1990; Hovring *et al.*, 1994).

Remarkably, TRF1 evolves rapidly (Broccoli *et al.*, 1997) and does not show significant amino acid identity with Rap1p, the major duplex telomeric DNA-binding protein of the yeasts *Saccharomyces cerevisiae* (Shore, 1994) and *Kluyveromyces lactis* (Larson *et al.*, 1994; Krauskopf and Blackburn, 1996). However, the yeast and mammalian telomeric proteins appear to be distantly related, since both carry Myb-related DNA-binding domains (Konig *et al.*, 1996). Rap1p contains two Myb repeats, which, separated by a 40 amino acid linker, dock onto two GGTGT sequences that are spaced by 3 bp. Since Rap1p and c-Myb bind DNA differently (Ogata *et al.*, 1994; Konig *et al.*, 1996), no *a priori* predictions can be made on the DNA-binding mode of TRF1 and 2. Indeed, the fact that TRF1 and 2 contain only a single Myb motif (Chong *et al.*, 1995; D.Broccoli, A.Smogorzewska, L.Chong and T.de Lange, in preparation) points to a crucial difference in the way these factors bind to DNA compared with c-Myb and Rap1p.

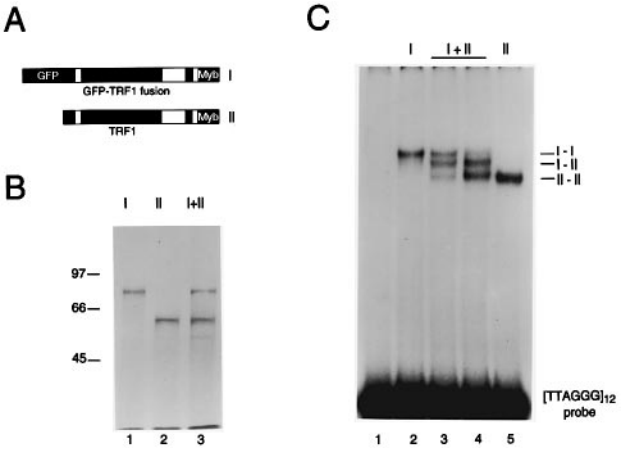

Fig. 2. hTRF1 binds DNA as a dimer. (**A**) Schematic representation of the two hTRF1 derivatives (I and II) that differed in size by ~26 kDa. Form I contains the 26 kDa GFP protein fused to the N-terminus of hTRF1. Form II contains an N-terminal addition of 43 amino acids encoded by polylinker sequences. (**B**) SDS–PAGE gel showing [^{35}S]methionine-labeled products resulting from *in vitro* translation of the hTRF1 derivatives depicted in (A). (**C**) Gel-shift assay with hTRF1 derivative I (lane 2), hTRF1 derivative II (lane 5) or a mixture of the two (lanes 3 and 4). The probe is a restriction fragment containing the sequence $[TTAGGG]_{12}$. For the reactions in lanes 3 and 4, the hTRF1 derivatives were produced by co-translation. The ratio of plasmids used in the coupled transcription–translation reaction was 1:1 for lane 3 and 1:2 (excess of hTRF1 derivative II) for lane 4. Lane 1 represents a reaction with mock *in vitro* translation product. The protein compositions of the gel-shift complexes are indicated to the right of the gel.

Here we report that human TRF1 binds to DNA as a dimer, thus suggesting that, like Rap1p and c-Myb, TRF1 contacts the DNA with two HTH motifs. Results obtained with the yeast two-hybrid assay in conjunction with *in vitro* DNA binding studies implicate the TRF-specific conserved domain in dimerization. We further extend the analogy between Rap1p and TRF1 by showing that TRF1, like Rap1p (Vignais and Sentenac, 1989; Gilson *et al.*, 1993; Muller *et al.*, 1994), bends DNA and binds along telomeric repeat arrays without strong cooperative interactions. Based on the conservation of this property in human and yeast telomeric proteins, we suggest that DNA bending is relevant to telomere function *in vivo*.

Results

hTRF1 binds telomeric DNA as a dimer

Since TRF1 harbors only a single Myb repeat, we asked whether it binds to DNA as a homodimer. We have shown previously that cloned TRF1 protein produced by *in vitro* translation in a rabbit reticulocyte lysate binds to DNA probes containing 12 telomeric tandem repeats (the optimal TRF1-binding site), resulting in a complex that co-migrates with hTRF1 purified from HeLa cells (Chong *et al.*, 1995). We employed this system to synthesize two hTRF1 derivatives of different size and study the gel-shift complexes formed by mixtures of these proteins, a strategy originally employed by Hope and Struhl to show dimerization for GCN4 (Hope and Struhl, 1987). A larger derivative of hTRF1 (I in Figure 2A) was created by fusing the 26 kDa green fluorescent protein (GFP) onto the N-terminus. As expected, *in vitro* translation of the GFP–TRF1 fusion and hTRF1 (II in Figure 2A) resulted in two

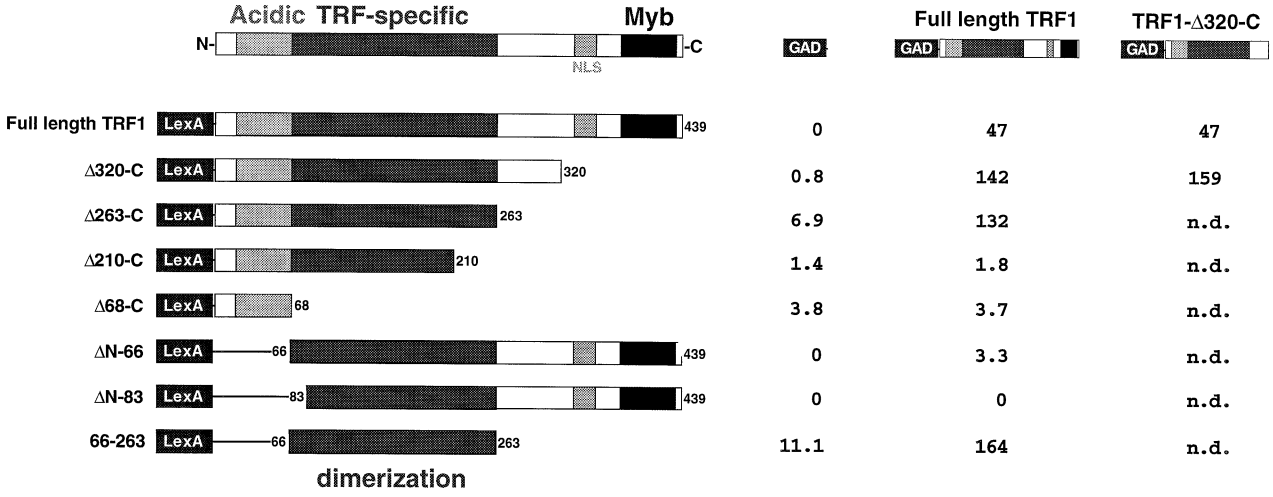

Fig. 3. Identification of the dimerization domain of hTRF1 using the two-hybrid system. β-Galactosidase levels were measured for strains containing plasmids expressing various LexA–TRF1 hybrid genes (as indicated) along with plasmids expressing either the GAL4 activation domain (GAD) or GAD fusions containing full-length or truncated (Δ320-C) TRF1. The values represent an average of three independent transformants. Values <0.01 are indicated by 0; n.d.= not determined.

polypeptides that differed by ~26 kDa in their apparent mol. wt (Figure 2B, lanes 1 and 2).

Both forms of hTRF1 were active for DNA binding and gave rise to gel-shift complexes of different migration behavior, with the larger protein creating a slower migrating complex (Figure 2C, lanes 2 and 5). When the two hTRF1 derivatives were co-translated (Figure 2B, lane 3), the same two gel-shift complexes were apparent (Figure 2C, lanes 3 and 4). In addition, a third complex was formed that migrated to an intermediate position in the native gel. This third complex was not observed in binding reactions with either of the hTRF1 derivatives alone (Figure 2C and data not shown), indicating that its formation depended on the presence of both proteins. Furthermore, the ratio of the three complexes was influenced by the ratio of the two plasmids added to the coupled *in vitro* transcription–translation system (Figure 2C). Since the third complex migrated in between the complexes observed with each hTRF1 derivative alone, it is likely to contain an intermediate protein mass. The simplest interpretation of these results is that hTRF1 binds to DNA as a dimer. According to this interpretation, the slowest migrating complex represents a homodimer of the GFP–TRF1 fusion, the fastest migrating complex represents a homodimer of hTRF1 and the middle complex represents a heterodimer formed by interaction of these two polypeptides. No gel-shift complexes were observed that could represent hTRF1 monomers. When both proteins were synthesized separately and incubated together, no formation of heterodimers could be demonstrated in subsequent DNA binding assays (data not shown), suggesting that hTRF1 dimers do not exchange subunits rapidly.

Dimerization is mediated by the TRF-specific conserved domain

To determine which sequences in hTRF1 are responsible for dimer formation, we employed the yeast two-hybrid system (Fields and Song, 1989). Co-expression of full-length hTRF1 fused to LexA (LexA–TRF1) and full-length hTRF1 fused to the GAL4 activation domain (GAD–TRF1) resulted in transcriptional activation of the *lacZ* reporter gene that was dependent on the hTRF1 sequences in both hybrids (Figure 3 and data not shown). Moreover, activation was not restricted to the LexA reporter system, since similar activation was observed when hTRF1 was fused to the GAL4 DNA-binding domain (data not shown).

We first determined whether the Myb repeat was required for TRF1–TRF1 interaction. Deletion of the C-terminal 119 amino acids of hTRF1 (LexAΔ320-C) from both the LexA– and the GAD–TRF1 hybrids did not diminish activation, indicating that the Myb domain was not required for interaction.

To define further the dimerization domain, a series of C- and N-terminal deletions of LexA–TRF1 was tested for interaction with GAD–TRF1. Deletion of the C-terminal 25% of TRF1 (LexAΔ263-C) had no effect on activation. In contrast, partial (LexAΔ210-C) or complete (LexAΔ68-C) removal of the conserved domain abolished the interaction with GAD–TRF1.

Deletion of the N-terminus of hTRF1 demonstrated that the acidic domain was not required for dimerization. LexAΔN-66 displayed a lower, but reproducible level of activation that was dependent on the presence of GAΔ–TRF1 and not found with GAD alone. Further deletion into the N-terminal region of the conserved TRF-specific domain (LexAΔN-83) completely abolished activation.

While each of the fusion proteins was stably expressed as determined by Western blotting (see Materials and methods), we cannot exclude the possibility that the lack of activity of LexAΔ210-C, LexAΔ68-C and LexAΔN-83 is due to misfolding of these deletion mutants.

The N- and C-terminal deletions suggested that the TRF-specific conserved domain was required for dimerization. To determine whether this part of hTRF1 was sufficient for the interaction, a LexA–TRF1 fusion protein containing only the conserved domain (LexA66–263) was co-expressed with GAD–TRF1. The resulting activation of the reporter gene demonstrates that the TRF-specific

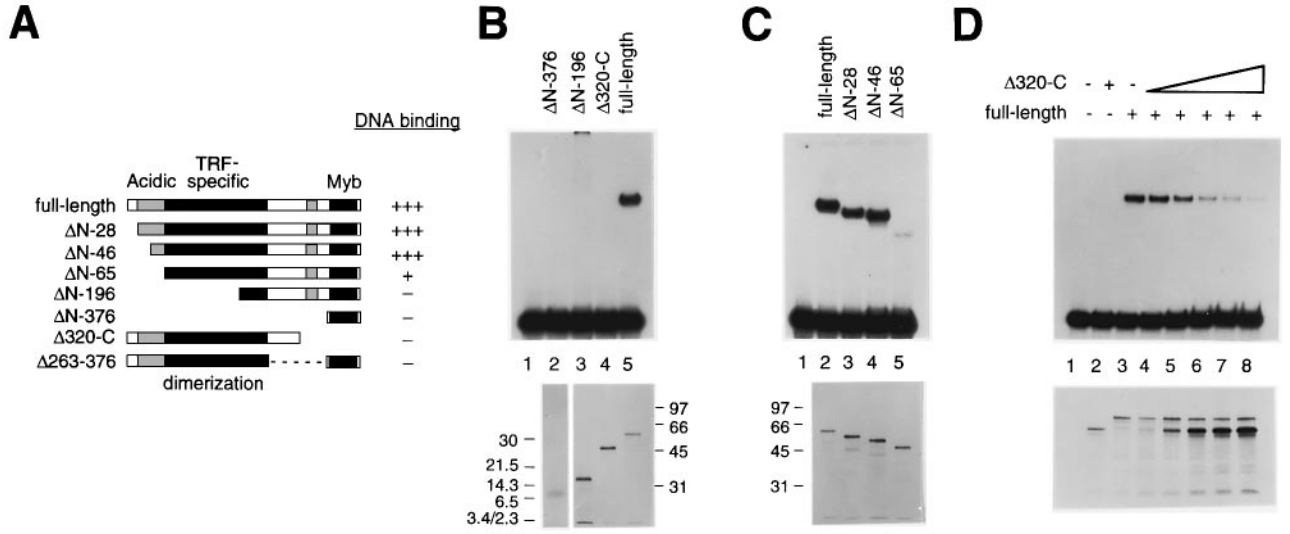

Fig. 4. Deletion mapping of the sequences in hTRF1 required for DNA binding. (**A**) Schematic of the deletion mutants used and summary of their DNA-binding activity. (**B**) and (**C**) Gel-shift reactions with the indicated hTRF1 derivatives. (**D**) Co-translation experiments showing that hTRF1 requires two Myb repeats for DNA binding. Increasing amounts of Δ320-C were co-translated with full-length hTRF1 and the mixtures were assayed for TTAGGG repeat-binding activity. The gel-shift probe in (B–D) is a $[TTAGGG]_{12}$-containing restriction fragment. To ensure that each protein was present at the same concentration, the proteins were synthesized in parallel in the presence of $[^{35}S]$methionine and the labeled products were analyzed on SDS–PAGE (shown below each of the gel-shift assays).

conserved domain is both necessary and sufficient for dimerization.

Certain LexA–TRF1 derivatives (specifically, LexAΔ263-C, LexAΔ68-C and LexA66–263) were found to activate transcription of the LexA reporter gene weakly, in a manner that is independent of hTRF1 sequences in the GAD fusion partner (Figure 3). Thus, both the acidic domain and the dimerization domain have some intrinsic ability to activate transcription in this context.

hTRF1 dimers require two Myb domains for DNA binding in vitro

hTRF1 deletion mutants were tested by gel-shift assay of *in vitro* synthesized proteins for their ability to bind to telomeric DNA (Figure 4). While full-length hTRF1 bound to a $[TTAGGG]_{12}$ probe in this assay (Figure 4B, lane 5), hTRF1 truncated at position 320 (Δ320-C) did not bind to DNA (Figure 4B, lane 4). Since this deletion removes the Myb domain, the lack of DNA binding with the Δ320-C truncation is consistent with the requirement of the Myb motif for interaction with the telomeric site. However, while the Myb domain is necessary for DNA binding, it does not appear to be sufficient for this activity, as shown by the lack of complex formation with deletions ΔN-196 and ΔN-376 (Figure 4B, lanes 2 and 3).

These results suggested that dimer formation is a prerequisite for DNA binding, as both ΔN-196 and ΔN-376 lack the dimerization domain as defined by the two-hybrid assay. In agreement with this view, removal of the first 28 or 46 amino acids did not affect the DNA-binding activity of hTRF1 (Figure 4C, lanes 3 and 4). In addition, ΔN-65 was clearly capable of DNA binding, albeit with diminished activity (Figure 4C, lane 5).

The requirement for dimerization could be explained if stable interactions with telomeric DNA depend on the coordinate binding of two Myb repeats. To test this possibility, we determined whether hTRF1 dimers need to contain both Myb repeats to bind to DNA. To this end, full-length hTRF1 was co-translated with increasing amounts of the ΔC-320 mutant under conditions known to generate heterodimers (Figure 4D). The resulting mixtures were found to contain a single DNA-binding activity, forming a complex that co-migrated with the full-length hTRF1 complex. We did not observe the second, smaller complex predicted to occur if the heterodimer lacking the second Myb motif could bind to DNA. Furthermore, as more mutant hTRF1 was synthesized in the reactions, the abundance of the hTRF1 gel-shift complex diminished (Figure 4D), as would be expected if heterodimers with only a single Myb motif failed to bind DNA. These results were consistent with the notion that two Myb motifs are required for the formation of a stable DNA–protein complex and indicated that this requirement is met by the formation of hTRF1 homodimers. The relative orientation of the two Myb motifs on the telomeric DNA is likely to be important, since a simple fusion of the dimerization domain onto the Myb domain did not result in active protein (D263–376, see Figure 4A and data not shown).

hTRF1 bends DNA

c-Myb, the plant transcription factor Myb.Ph3 and Rap1p each induce a bend in their target site (Vignais and Sentenac, 1989; Gilson *et al.*, 1993; Muller *et al.*, 1994; Saikumar *et al.*, 1994; Solano *et al.*, 1995). In order to determine whether hTRF1 shares this feature, we employed an approach analogous to the circular permutation assay developed by Wu and Crothers (1984). To generate probes for this assay, we used PCR amplification to produce five DNA fragments of equal length, each harboring a hTRF1-binding site at a different position relative to the ends of the molecule (Figure 5A). Using this strategy on three similar plasmid templates with variable TTAGGG repeat array lengths (Figure 5A), three sets of permuted probes were generated which carried three, six or 12 tandem

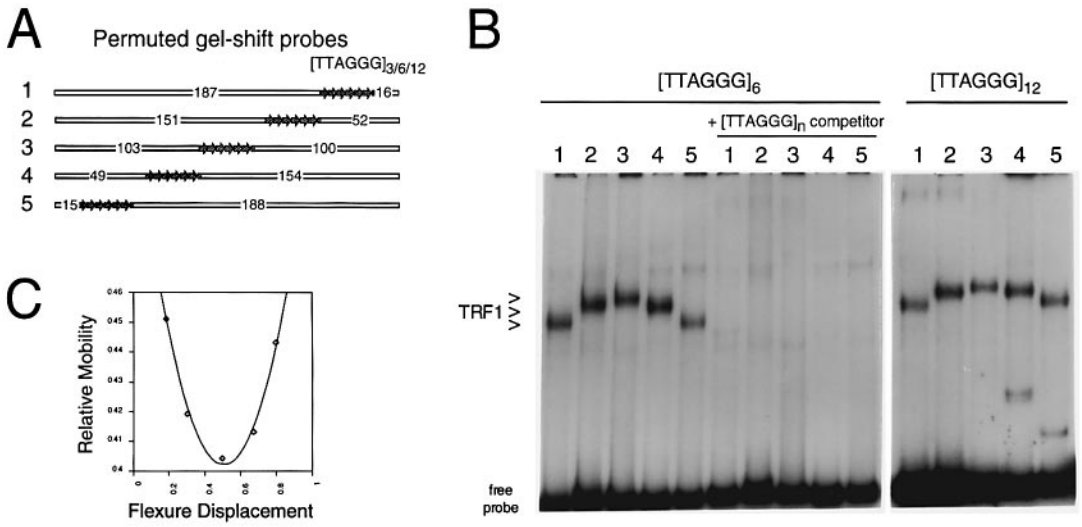

Fig. 5. hTRF1 bends DNA. (**A**) Schematic representation of two sets of five PCR-generated permuted gel-shift probes carrying either three, six or 12 complete tandem TTAGGG repeats. The length of the non-telomeric sequences in each of the probes is indicated. (**B**) Gel-shift assay with partially purified HeLa TRF1 and the labeled probes shown in (A). The assay on the left side was performed with probes containing $[TTAGGG]_6$ sites with or without added unlabeled $[TTAGGG]_n$ competitor DNA as indicated. The assay on the right hand side was performed with probes containing $[TTAGGG]_{12}$ sites. (**C**) Plot of the relative mobility (mobility of bound DNA/mobility of free DNA) of each of the TRF1 complexes against the flexure displacement in each probe. The data points represent probes 1–5 from right to left; values on the *x*-axis indicate the distance from the middle of the TTAGGG repeat site to the 5′ end of the probe divided by the length of the probe. The data points were interpolated with the function derived by Ferrari *et al.* (1992).

TTAGGG repeats (referred to as 3mer, 6mer and 12mer probes).

Labeled DNA probes were incubated with purified HeLa hTRF1 under conditions in which one hTRF1 dimer binds per probe molecule (Zhong *et al.*, 1992) and the mobility of the resulting complexes was analyzed on native polyacrylamide gels. The permuted sets of fragments had the same electrophoretic mobility as expected from their equal lengths. Complexes were formed with each of the permuted 6mer and 12mer probes with approximately the same efficiency, and this binding was specific as demonstrated by competition with a plasmid carrying an array of TTAGGG repeats (Figure 5B and data not shown). As shown in Figure 5B for the 6mer and 12mer probes, an effect of the position of the hTRF1-binding site within the probes was observed. Slower migrating complexes were obtained when the binding site for hTRF1 was located more centrally in the DNA molecule, consistent with the induction of DNA bending upon hTRF1 binding. A similar anomalous migration pattern indicative of bent DNA was observed with the hTRF1 complexes formed on the set of 3mer probes (data not shown) but, in agreement with a previous report (Zhong *et al.*, 1992), the binding was very weak.

To determine the locus and extent of DNA bending, we plotted the relative mobility of each hTRF1–DNA complex against the flexure displacement, and these data points were interpolated with a quadratic function (Ferrari *et al.*, 1992) to derive an estimate of the deviation from linearity (Figure 5C and data not shown). Values ranging from 64 to 66° were found in five experiments with the 6mer set and similar values of 57° and 59° resulted from two experiments with the 12mer probes, indicating that hTRF1 induced a shallow distortion in which the DNA deviates from linearity by ~60°. A similar bending angle was deduced when the equation derived by Thompson and Landy was used (Thompson and Landy, 1988). The minimum of the parabola maps the site of bending to ~2 bp 5′ of the center of the TTAGGG repeat arrays in both sets of probes. Since it is not known where hTRF1 binds within the TTAGGG repeat arrays, we cannot determine where this bend is in relation to the position of hTRF1 in the probes.

In some cases, the shape of the protein itself, rather that a protein-induced bend, is thought to be responsible for the anomalous migration of DNA–protein complexes in the circular permutation assay (Gartenberg *et al.*, 1990). We therefore sought independent evidence that hTRF1 distorts its binding substrate and employed the circularization assay for this purpose (Kotlarz *et al.*, 1986). Since the rate of intramolecular ligation of small DNA fragments is affected by the presence of a natural or protein-induced DNA bend, we determined the effect of hTRF1 on circularization of a 217 bp restriction fragment containing 27 tandem TTAGGG repeats. The reaction was monitored by gel electrophoresis of samples that were treated with T7 gene 6 exonuclease to facilitate identification of the exonuclease-resistant ligation product representing the circular form of the 217 bp fragment. In three independent experiments, the appearance of the circular ligation product was enhanced when active baculovirus-derived hTRF1 was added to the reactions, and the formation of the circle depended on the concentration of the hTRF1 protein in the reactions (Figure 6A). At the highest protein concentrations, the enhancing effect of hTRF1 is partially lost, possibly because the binding of multiple hTRF1 dimers to one DNA molecule cancels out the bending angles. No enhancement was observed when the hTRF1 protein was heat inactivated for 30 min at 55°C before addition to the reactions (Figure 6B). In addition, no enhancement occurred with a 192 bp fragment that does not contain TTAGGG repeats, indicating that the effect is

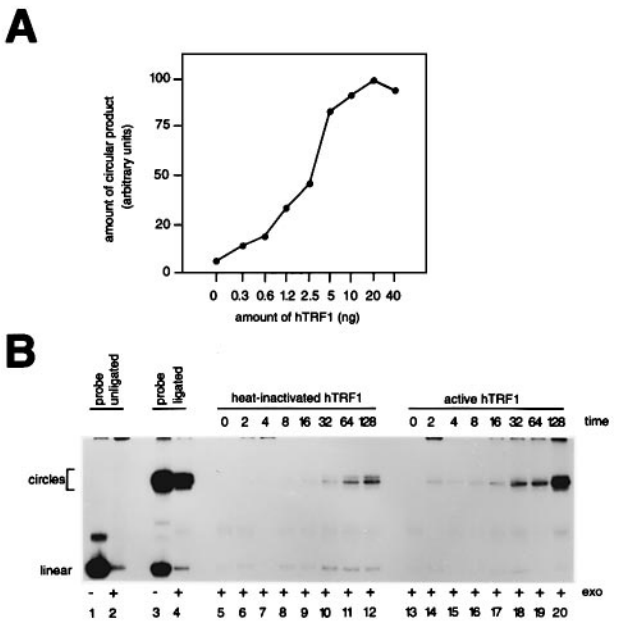

Fig. 6. hTRF1 enhances DNA cyclization. (**A**) Effect of increasing amounts of baculovirus-expressed hTRF1 on cyclization of a 217 bp DNA fragment containing 27 TTAGGG repeats. (**B**) The rate of cyclization of the 217 bp DNA fragment was measured in the presence of either heat-inactivated (lanes 5–12) or active (lanes 13–20) baculovirus-expressed hTRF1. Ligation time (in minutes) is indicated over lanes 5–20. Exonuclease digestion was performed as indicated prior to loading of samples in order to eliminate linear ligation products. Lanes 1 and 2 show unligated samples. In lanes 3 and 4, the fragment was ligated with a 20-fold higher concentration of ligase as compared with samples 5–20. Lane 4 is under-loaded due to loss of DNA after exonuclease digestion.

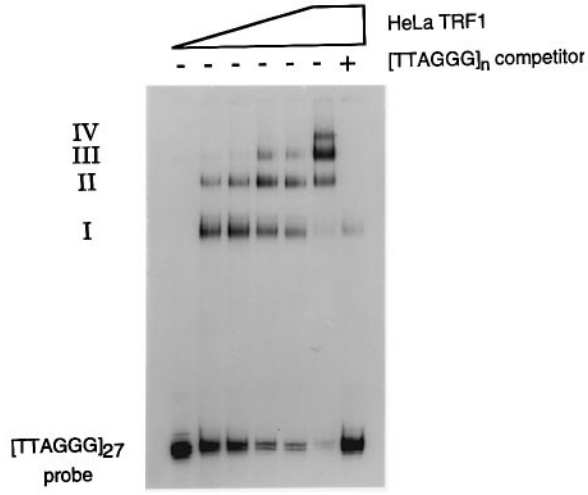

Fig. 7. hTRF1 dimers do not show strong cooperative interactions. Increasing amounts (1, 2, 3, 4 and 8 μl) of partially purified HeLa TRF was added to a labeled probe derived from plasmid pTH5 (de Lange *et al.*, 1990) which carries 27 tandem TTAGGG repeats. Complexes containing 1–4 TRF1 dimers are identified to the left of the gel (Roman numerals). The first lane is a mock reaction in the absence of hTRF1. Unlabeled TTAGGG repeat competitor DNA was added to the reaction in the last lane.

due to hTRF1 binding to its telomeric site (data not shown). The extent to which hTRF1 enhanced the rate of circularization was determined with (Figure 6B) or without (data not shown) prior treatment with exonuclease. In three experiments, TRF1 was found to enhance circularization by 8- to 16-fold at early time points. At later time points (>1 h), the effect was less strong (2-fold) possibly because hTRF1 is inactivated in the reactions. Rate measurements using the 192 bp control fragment that lacked a hTRF1-binding site showed that TRF1 did not have a non-specific effect on the rate of DNA circularization (data not shown). These results are consistent with the notion that hTRF1 induces a bend in telomeric DNA.

hTRF1 dimers bind along TTAGGG repeat arrays without strong cooperativity

We asked how hTRF1 interacts with long arrays of TTAGGG repeats that represent more closely the extended tracts of telomeric repeats at human chromosome ends. Gel-shift experiments were performed with a DNA probe containing an array of 27 telomeric repeats and increasing amounts of partially purified hTRF1 from HeLa nuclear extract. As more protein was used in the reactions, larger complexes were observed (Figure 7) which increased in size in four incremental steps, consistent with the acquisition of four dimeric hTRF1 units by the $[TTAGGG]_{27}$ probe. It is not excluded that this probe can accommodate additional hTRF1 dimers; such higher order complexes might not be resolved easily by the gel system used in these experiments. Thus, consistent with previous results and the binding of hTRF1 to probes with three TTAGGG repeats (Zhong *et al.*, 1992), the binding of four hTRF1 dimers to a $[TTAGGG]_{27}$ probe argues that the minimal hTRF1-binding site is not larger than seven tandem repeats. However, other approaches will be required to establish the actual size of the hTRF1 recognition site and the maximum density with which hTRF1 can bind along duplex telomeric DNA.

The ability of hTRF1 to interact with itself to form dimers raised the possibility that hTRF1 might display cooperative interactions when binding along the length of long telomeric tracts. However, the recruitment of additional hTRF1 dimers to hTRF1–DNA complexes does not appear to be strongly enhanced compared with binding to the free probe (Figure 7 and data not shown). The appearance of additional bound units of hTRF1 with increasing amounts of HeLa nuclear extract seems to be progressive and gradual. Note, for example, the persistence of complex II (containing two hTRF1 dimers) throughout the titration. Thus, no evidence was found for strong cooperative interactions on these and other probes with long telomeric arrays.

Discussion

This study revealed several novel features of TRF1 that are relevant to its function at mammalian telomeres. Human TRF1 was found to form a homodimer through interactions involving the TRF-specific, conserved domain in the N-terminal half of the protein. Dimerization was a prerequisite for DNA binding, presumably because it brings together two copies of the second domain conserved in human and mouse TRF1, the Myb-related DNA-binding motif. In addition, TRF1 was found to form extensive protein arrays along the telomeric DNA, and binding of

TRF1 induced a shallow bend in its telomeric site. These results reveal striking similarities between TRF1 and the yeast telomeric protein Rap1p and argue that these proteins may have a previously unappreciated architectural role at telomeres in yeasts and mammals.

Whereas the majority of Myb-related DNA-binding proteins carry two or three Myb repeats, the TRF proteins belongs to the class of Myb proteins that harbor only a single Myb motif. Here we show that TRF1 binds as a homodimer, thus creating an overall architecture that is functionally similar to other Myb proteins in the sense that two Myb repeats are linked in one protein. In addition, we found that, similar to what is seen with c-Myb and Rap1p (Henry *et al.*, 1990; Saikumar *et al.*, 1990), both Myb repeats in the TRF1 dimers are required for DNA binding, indicating a unifying theme for Myb-related DNA-binding proteins: the use of a pair of HTH motifs to recognize DNA. Since its primary sequence indicates that TRF2 has a similar domain structure (D.Broccoli, A.Smogorzewska, L.Chong and T.de Lange, in preparation), it is likely that this theme of twin Myb repeats juxtaposed on DNA by dimerization extends to this telomeric protein.

Our results suggest that dimerization may also play a role in DNA site recognition by other single Myb repeat proteins, such as Tbf1p, IBP, BFP-1, MybSt1, Adf1 and CHD1 (England *et al.*, 1991; Liu and Tye, 1991; da Costa e Silva *et al.*, 1993; Baranowskij *et al.*, 1994; Lugert and Werr, 1994; Stokes and Perry, 1995). Interestingly, many of these proteins have been shown to interact with a DNA recognition sequence that features direct repeats, consistent with a DNA-binding mode in which homodimerization positions two identical HTH motifs in contact with tandemly repeated sites. These considerations raise the possibility that single Myb repeat proteins in general may interact with direct repeats.

While in c-Myb the two HTH motifs come in direct contact with each other, contacting a single short site in the major groove, in Rap1p the two Myb repeats are separated by a linker, contacting two distinct, directly repeated sites. Since c-Myb and Rap1p clearly have different interactions with their recognition sites, it is not possible to predict the structure of the TRF1–DNA complex at this stage. Nevertheless, it is tempting to speculate that the binding of tandem repeats by TRFs is a direct reflection of the presence of two identical recognition helices in the dimers and that the two HTH motifs are used independently in contacting adjacent repeats. Several examples of homodimeric factors that bind to direct repeats have been reported in both yeast (e.g. HAP1, Zhang and Guarente, 1994) and higher eukaryotes (e.g. RAR, Towers *et al.*, 1993). These factors dimerize through symmetrical protein–protein interactions, and their ability to bind to direct repeats is attributed to free swiveling of the DNA-binding domain around a flexible linker. It is possible that the poorly conserved domain of TRF1 located between the dimerization domain and the Myb repeat similarly functions as a flexible hinge region.

The finding that TRF1 dimerization occurs in the yeast two-hybrid system indicates that TRF1 dimerizes independently of its binding to telomeric DNA. Further evidence for such pre-formed TRF1 dimers was obtained from the fractionation of HeLa-derived hTRF1 on a SuperDex gel filtration column on which TRF1 migrates as a 100–120 kDa protein (J.Feng and T.de Lange, unpublished data), consistent with a homodimer of the 50 kDa hTRF1 polypeptide. We have been unable to observe the exchange of subunits between TRF1 dimers, suggesting that, once formed, TRF1 dimers may be relatively stable.

Bilaud *et al.* (1996) have shown that the isolated Myb repeat domains of both TRF1 and TRF2 can bind TTAGGG repeats in a SouthWestern assay. While it is not clear that the binding activity of these fragments is similar to that of full-length protein, it seems likely that in the SouthWestern assay the attachment of the Myb domains to a solid matrix can (at least partially) substitute for the requirement for dimerization. A second possibility is that the isolated Myb domain of the TRF proteins can form a complex with DNA under conditions of high DNA and/or protein concentration.

Each of the activities of TRF1 described here, binding to DNA with two Myb repeats, absence of strong cooperative interactions and DNA bending, are also seen with Rap1p, the major duplex telomeric DNA-binding protein in yeast (reviewed in Smith and de Lange, 1997). The resemblance of TRF1 to Rap1p is particularly striking because the primary sequences of these proteins are not similar, indicating that biochemical features of these telomeric proteins are conserved even as their primary sequences evolve rapidly. We favor the interpretation that the identified properties of these telomeric proteins are conserved because they reflect key aspects of their function at telomeres.

DNA bending by telomeric proteins could induce a higher order structure at telomeres that is required for their function. It is noteworthy that human telomeres appear to be very compact structures when visualized by immunogold electron microscopy (Ludérus *et al.*, 1996), suggesting that some protein is responsible for their tight packaging in interphase nuclei. The ability of TRF1 to bend DNA could contribute considerably to the overall configuration of the telomeric DNA. Although a single TRF1 dimer induced only a minor distortion *in vitro*, the acquisition of as few as three TRF1-binding units along the telomeric tracts could result in the folding back of the telomere on itself. Thus, TRF1 binding could drastically alter the overall structure of the telomeric complex in a manner that is important for telomere function. TRF1 was recently shown to control telomere length in human cells (van Steensel and de Lange, 1997) and duplex telomeric DNA-binding proteins in yeasts have been implicated in telomere length regulation (Conrad *et al.*, 1990; Lustig *et al.*, 1990; McEachern and Blackburn, 1995; Zakian, 1995a; Krauskopf and Blackburn, 1996; Cooper *et al.*, 1997), in suppression of telomere–telomere recombination (Li and Lustig, 1996), in telomeric silencing (Kyrion *et al.*, 1992; Shore, 1994; Cooper *et al.*, 1997) and in telomere function in meiosis or sporulation (Cooper *et al.*, 1997). Each of these aspects of telomere function may well depend on a critical configuration of the telomeric complex achieved (in part) via DNA distortions.

Materials and methods

Coupled in vitro transcription–translation

hTRF1 deletion mutants used for the *in vitro* coupled transcription–translation experiments were cloned in the vector pET28(a) (Promega)

in the *Nco*I and *Eco*RI sites using PCR-generated fragments. The GFP–TRF1 fusion product was cloned in pBluescriptKS+. PCR-directed mutagenesis was used to eliminate from this construct the start codon of the hTRF1 gene by mutating it from ATG to ATT in order to suppress the occurrence of internal translation at this position. The GFP sequence was obtained from pS65T-C1 (Clontech). Expression of TRF1 derivatives was achieved by using a rabbit reticulocyte lysate system (Promega) using reaction conditions essentially as described by the supplier. Briefly, between 0.2 and 1 μg of total plasmid DNA was used per 20 μl reaction containing T7 RNA polymerase in the presence of [^{35}S]methionine (to visualize products on SDS–PAGE) or without labeled amino acids (for gel-shift assays). After the transcription–translation reaction, samples were diluted 1:5 with the addition of 80 μl of buffer D (Chong *et al.*, 1995). Of this mixture, 0.5–5 μl was used in gel-shift reactions.

Gel-shift assays

Gel-shift assays were performed as described previously (Zhong *et al.*, 1992) using labeled restriction fragments as probes. Most of the experiments were performed with a 142 bp *Hin*dIII–*Asp*718 fragment from the plasmid pTH12 (Zhong *et al.*, 1992), which contains 12 tandem TTAGGG repeats. In addition, an *Eco*RI fragment from pTH5 (de Lange *et al.*, 1990) containing 27 tandem TTAGGG repeats was employed. Competitions were executed with pTH5. The source of hTRF1 was either *in vitro* translation product (above) or HeLa TRF1 purified over P11, DEAE, CM–Sepharose, a column containing *Escherichia coli* chromosomal DNA and a column containing TTAGGG repeat DNA (Chong *et al.*, 1995). All detectable TTAGGG repeat-binding activity in this fraction could be supershifted with a TRF1-specific antibody that does not react with TRF2 (Ludérus *et al.*, 1996).

Yeast two-hybrid analysis

LexA–TRF1 hybrids were generated by PCR amplification of DNA sequences encoding the indicated amino acids from a plasmid containing the full-length hTRF1 cDNA (phTRF1.4.7, Chong *et al.*, 1995) followed by insertion into the *Eco*RI and *Bam*HI sites of vector pBTM116 (Bartel *et al.*, 1993). GAD–TRF1 hybrids were generated similarly using vector pACT2 (Clontech). All fusion proteins contained a few additional amino acids (encoded by vector linker sequences) at their carboxy-termini. Expression of the LexA–TRF1 fusion proteins was verified by Western blotting using an anti-LexA antibody.

Two-hybrid experiments were performed in the yeast strain L40 (*MATa his3D200 trp1-901 leu2-3, 112ade2 LYS::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-lacZ*) (Hollenberg *et al.*, 1995). β-Galactosidase activities were measured essentially as described (Guarente, 1983) except that cells were disrupted by freeze–thawing using liquid N_2. The average value of three individual transformants for each set of plasmid constructs is reported. Values from individual transformants differed by <30% from the average.

DNA bending assay

PCR primers were used to generate DNA fragments with the composition indicated in Figure 5 using pTH3, pTH6 and pTH12 (Zhong *et al.*, 1992) as templates, which contain three, six and 12 tandem TTAGGG repeats, respectively. The products were end-labeled with [γ-^{32}P]ATP and polynucleotide kinase and the labeled fragments were isolated by preparative acrylamide gel electrophoresis. Gel-shift reactions with partially purified HeLa hTRF1 were performed as described above, and the migration of the complexes was analyzed as described by Ferrari *et al.* (1992) and Thompson and Landy (1988).

Expression of hTRF1 in insect cells

An N-terminally $[His]_6$-tagged version of hTRF1 was cloned in the baculovirus expression vector pBacPak8 (Clontech). This vector was used to co-transfect insect Sf21 cells together with linearized baculovirus BacPak6 (Clontech). Recombinant viruses were plaque purified, screened for hTRF1 expression and amplified. For protein production, a 100 ml suspension culture of Sf21 cells was infected at an m.o.i. of ~10 p.f.u./cell and harvested after 40 h. Cells were washed twice in phosphate-buffered saline and resuspended in 4 μl of 5 μM imidazole, 500 mM NaCl, 20 mM Tris–HCl pH 7.9. After sonication, the extract was centrifuged on a SW55 rotor at 20 000 r.p.m. for 20 min at 4°C. The supernatant was filtered through a 0.45 μm filter and applied batch-wise to 400 μl (settled volume) of Ni-charged Sepharose resin (Pharmacia). After extensive washing of the resin, hTRF1 was eluted with 2 ml of 1 M imidazole, 500 mM NaCl and 20 mM Tris–HCl pH 7.9. The purified protein was dialyzed against buffer D containing 300 mM KCl (Chong *et al.*, 1995). As judged from Coomassie staining, the resulting hTRF1 protein appeared to be 95–99% pure.

Circularization assay

An *Asp*718-cut kinase end-labeled 217 bp DNA fragment containing an array of 27 TTAGGG repeats was used. The DNA was incubated for 20 min at room temperature either with active or heat-inactivated (55°C for 30 min) baculovirus-expressed hTRF1 in 20 mM HEPES-KOH pH 7.9, 200 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol. For the hTRF1 titration experiments (Figure 6A), reactions were carried out with 35 ng/ml of DNA and hTRF1 protein concentrations that varied from 15 to 2000 ng/ml. For rate measurements (Figure 6B), the DNA concentration was 40 ng/ml and hTRF1 was added to 500 ng/ml. ATP was added to 1 mM and ligase to 10 U/ml (protein titration) or to 1000 U/ml (rate measurements). Ligation reactions were performed at 23°C and allowed to proceed for 30 min (protein titration) or from 0 to 128 min (rate measurements). Reactions were stopped by the addition of $\frac{1}{2}$ volume of stop buffer (75 mM EDTA, 3 mg of proteinase K/ml, 15% glycerol) and incubation at 55°C for 15 min. Exonuclease-treated samples were phenol extracted, ethanol precipitated, resuspended in 20 μl of 40 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl and 50 U of T7 gene 6 exonuclease (USB) and incubated for 2 h at 37°C. Digestions were terminated by the addition of $\frac{1}{2}$ volume of stop buffer and incubation at 55°C for 15 min. Samples were run on 6% polyacrylamide gels in TBE. Quantitation of products was obtained by PhosphorImager (Molecular Dynamics).

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