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ST-1, a 39-Kilodalton Protein in *Trypanosoma brucei*, Exhibits a Dual Affinity for the Duplex Form of the 29-Base-Pair Subtelomeric Repeat and Its C-Rich Strand

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In our attempt to identify telomere region-binding proteins in *Trypanosoma brucei*, we identified ST-1, a polypeptide with novel features. ST-1 was chromatographically purified from S-100 cell extracts and was renatured from a sodium dodecyl sulfate-protein gel as a 39-kDa polypeptide. It forms a specific complex with the trypanosome telomere repeats of TTAGGG, but more significantly, it shows a higher affinity for the 29-bp subtelomere repeats of *T. brucei*. These 29-mer boxes are a large tandem series of telomere-derived repeats which separate the simple telomere DNA from middle-repetitive telomere-associated sequences on many chromosomes. ST-1 is the first example of a protein binding within such large repetitive subtelomere elements in trypanosomes or other organisms. ST-1 is also novel in that it has a selective affinity for the C-rich strands of both the subtelomeric 29-mer and the telomere repeats, comparable to that for the duplex form of the respective repeats. All previously described telomere-binding proteins have affinity for only the duplex form or for the G-rich strand. This C-rich strand binding specificity of ST-1 may provide insight into this protein's mechanism of binding in vivo.

The telomeric region forms the physical end of chromosomes and plays a crucial role in the biology of eukaryotic organisms. Not only does it stabilize chromosomes and allow their complete replication, but it is also thought to have a role in cell senescence (for reviews, see references 2 and 52), and it downregulates expression of juxtaposed genes (1, 24).

In *Trypanosoma brucei*, the protozoan flagellate responsible for African sleeping sickness, the telomeric region is particularly interesting. First, it is the site of antigenic variation, a process in which genes encoding the variable surface glycoprotein (VSG) coat of the bloodstream form of the organism transpose to various telomeric regions and then are activated, one at a time, allowing the parasite to evade the host immune system. Five to 20 different telomeres have been identified as expression sites for the VSG genes (for reviews, see references 4 and 14). Another possibly related and unique feature of trypanosome telomeres is an unusually modified nucleotide present in the terminal regions of all chromosomes except the active VSG expression site. This modification is differentially regulated, being found only in the bloodstream and not the procyclic life form, and is specific to trypanosomes that undergo antigenic variation (23). African trypanosomes also possess a unique set of 100 minichromosomes which range in size from 50 to 100 kb. They are the main source of telomeres in the cell and evidently consist largely of repeated sequences, some with VSG genes near their telomeres (49).

Proteins that bind telomere repeat sequences (e.g., TT GGGG in a *Tetrahymena* species and TTAGGG in trypanosomes and humans) have been identified in a variety of organisms and are thought to be related to the protection, stability, replication, and/or architecture of chromosome ends. One class of factors interacts with the 3′ G-rich strand overhang, which in several organisms consists of two copies of the telomere repeat. This class includes telomerase (reference 3 and references therein), a ribonucleoprotein enzyme that synthesizes these G-rich telomere repeats, and the α/β telomere proteins in *Oxytricha nova*, which cap the tips of chromosomes and the β subunit of which induces G-quartet formation in vitro (a four-stranded structure formed by G-G stacking [references 26 and 20 and references therein]), and α/β-equivalent proteins in *Stylonychia mytilis* (19), *Euplotes crassus* (48), and *Xenopus laevis* (9) and possibly also birds (27). A second class of telomere-specific proteins that binds the double-stranded form of the telomere repeats has been identified in *Physarum polycephalum* (13) and mammalian cells (53). The best-characterized member of this group is RAP1, an essential protein in *Saccharomyces cerevisiae*, which binds yeast telomeres in vivo (11) and affects both telomere length and the expression of genes in the proximity (8, 30, 43). Recently, heterogeneous ribonucleoprotein-type proteins from mouse liver (34) and HeLa cells (28) were shown to bind the RNA equivalent of telomere repeats of TTAGGG with high affinity, but the significance of this result to telomere function remains to be determined. Notably, no proteins that bind the C-rich strand of the telomere repeat have yet been reported.

T. brucei and many other organisms share an organization at the telomeres. Middle-repetitive elements called telomere-associated sequences (TASs) are found proximal to the simple telomere repeats and are thought to undergo multiple recombination events that occur in the telomeric regions of chromosomes, such as exchange of DNA between nonhomologous chromosomes (human [references 17 and 50 and references therein]), meiotic recombination (human [42]), and chromosome size polymorphisms (*Plasmodium berghei* [12, 39] and tomato [7]). In *S. cerevisiae* (reference 33 and references therein), recombination of such TASs can rescue the cells from an otherwise lethal defect in which the chromosomes cannot replicate their normal terminal telomere repeats. A prominent example of such TAS-related rearrangement events, however, is the diversification of the subtelomeric VSG genes that is the basis of antigenic variation in African trypanosomes.

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TABLE 1. Inhibition of binding to Tel-ds and Subtel-ds by double-stranded DNAs[a]

DNA competitor	Sequence	Inhibition of binding to:	
		Tel	Subtel
Tel-ds	5′-GATCTTAGGGTTAGGGTTAGGGGATC-3′ 3′-CTAGAATCCCAATCCCAATCCCCTAG-5′	+	− (+)
Subtel-ds	5′-AGCTTTCGGGTTAGGGTGTTTCGGGTTAGCGGGAGCT-3′ 3′-TCGAAAGCCCAATCCCACAAAGCCCAATCGCCCTCGA-5′	+	+
R-Tel	3′-CTAGTGAGTGTGAGTGTGAGTGCTAG-5′ 5′-GATCACTCACACTCACACTCACGATC-3′	−	−
MycA	5′-ATC[T] CTTTAACAGATTTGTATTTAAGAATTGTTTTT-3′ 3′-TAG[A]$_{10}$GAAATTGTCTAAACATAAATTCTTAACAAAAA-5′	−	−
MycB	5′-GCTCAGATCTCCATGGAGCAAAAGCTCATTTCTGAAGAGGACT TGAATTAGATCTCCGT-3′ 3′-CGAGTCTAGAGGTACCTCGTTTTCGAGTAAAGACTTCTCCTGAAC TTAATCTAGAGGCA-5′	−	−
CEBP	5′-TGAGAAATTTCTATTAAAAACATGA-3′ 3′-ACTCTTTAAAGATAATTTTTGTACT-5′	−	NT
NS-B	5′-GATCCCCCTCGCACCGCGGAGATC-3′ 3′-CTAGGGGGAGCGTGGCGCCTCTAG-5′	−	NT
pTel 12	5′-TCGACGGTATCGATAAGCTTGAT[CCCTAA] ATCG-3′ 3′-AGCTGCCATAGCTATTCGAACTA[GGGATT]$_{12}$TAGC-5′	NT	−
Bgl II-LK	5′-[CAGATCTG]-3′ 3′-[GTCTAGAC]$_n$-5′	−	NT
pBR322 cleaved with *Hae*III, *Hpa*II, *Alu*I, and *Sau*3AI		−	−

[a] Various double-stranded DNAs were used as unlabeled competitors for binding to either the telomere (Tel) or the subtelomere (Subtel) repeat. +, competition; −, no competition; NT, not tested. The plus sign in parentheses indicates partial inhibition; brackets around parts of sequences indicate repeated sequences.

In many organisms, immediately proximal to the telomere repeats and separating them from the TASs is a series of tandem telomere-derived repeats (trypanosomes [45, 49], *Chlamydomonas reinhardtii* [38], *Arabidopsis thaliana* [41], and barley [29]). These subtelomere repeats are 29 bp in *T. brucei* and 27 bp in *P. berghei*. They delineate the border of subtelomeric rearrangement events involving the TASs (37, 40). It has been proposed that these telomere-variant repeats are not degenerate remnants of old telomeres but form a highly specialized structure between the telomere simple DNA and the TASs (37).

Given the complexity of interesting events that take place in the trypanosome telomeric region and the fact that trypanosome telomere-specific proteins had not yet been characterized, we examined telomere region-specific proteins of *T. brucei*. We isolated a 39-kDa polypeptide, ST-1, which binds specifically to the trypanosome telomere repeats. Notably, ST-1 showed an even higher affinity for the *T. brucei* 29-bp subtelomere repeat, making it the first polypeptide isolated with binding specificity for such repeats in any system. ST-1 is also novel in showing a C-strand preference, suggesting that it may act on telomere or subtelomere regions that are partially single stranded in this organism.

MATERIALS AND METHODS

Cells, extracts, oligonucleotides, and binding assays. Insect form *T. brucei* (procyclic, TREU667 strain) was grown in vitro under established conditions (15). Mammalian-form trypanosomes (bloodstream, ILTAT 1.3 strain) were propagated in rats and purified on DE-52 columns (18). S-100 extracts from parasites in both developmental stages were made as described previously (35), with the exception of the pH of the *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (7.5 instead of 7.9). Protein concentrations were measured by the Bradford assay (Bio-Rad). The radiolabeled telomere (Tel-ds) and subtelomere (Subtel-ds) repeat oligomers were prepared by annealing the synthesized homologous strands, Tel-C to Tel-G and Subtel-C to Subtel-G (see Table 2) and then labeling with the Klenow fragment of DNA polymerase and radiolabeled dATP. The duplex radiolabeled DNAs were gel purified to free them from any residual single-stranded DNA. To prepare the unlabeled double-stranded competitors, the appropriate oligomers were annealed, the ends were filled in with unlabeled deoxynucleoside triphosphates by using the Klenow fragment of DNA polymerase, and the double-stranded DNAs were then gel isolated.

For binding, 20 fmol of substrate radiolabeled oligonucleotide (Tel-ds or Subtel-ds) was incubated with 0.2 to 5 μg of protein (procyclic extracts unless specified in the figure legends) in 20 μl of binding buffer (25 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol) and 100 mM KCl (unless otherwise indicated), for 20 min at room temperature in the presence of 100 ng of poly(dA-dT). The resultant complexes were then resolved on a 4% native polyacrylamide gel (1:20 bis-to-acrylamide ratio) in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) at room temperature.

For competition assays, the extract was preincubated with unlabeled competitor oligonucleotides for 20 min before the radiolabeled oligonucleotide was added and allowed to bind for an additional 30 min. The sequences of the examined competitor oligonucleotides are included in Tables 1 and 2.

To prepare the in vitro-synthesized RNAs corresponding to the subtelomere repeat, Subtel-C and Subtel-G were annealed and inserted as a single copy at the *Hin*dIII site of pBluescript II KS plasmid (Stratagene), to create the construct pSubtel-1. T3 and T7 RNA polymerase runoffs were then made (method provided by the manufacturer) by using plasmid pSubtel-1 linearized with *Eco*RV and *Cla*I, respectively.

Chromatography and renaturation. S-100 trypanosome extract (20 mg of protein) was loaded on an Affi-Gel Blue column (10 ml) equilibrated with

TABLE 2. Inhibition of binding to Subtel-ds by single-stranded oligomers[a]

Competitor[b]	Sequence	Inhibition
Tel-C	5′-GATCCCCTAACCCTAACCCTAA-3′	− (+)
Tel-G	5′-GATCTTAGGGTTAGGGTTAGGG-3′	−
Subtel-C	5′-AGCTCCCGCTAACCCGAAACACCCTAACCCGAA-3′	+
Subtel-G	5′-AGCTTTCGGGTTAGGGTGTTTCGGGTTAGCGGG-3′	−
R-Tel-C	5′-GATCACTCACACTCACACTCAC-3′	−
R-Tel-G	5′-GATCGTGAGTGTGAGTGTGAGT-3′	−
$MycA_u$	5′-ATCTTTTTTTTTCTTTAACAGATTTG-3′	−
$MycA_1$	5′-AAAAACAATTCTTAAATACAAATCTGTT-5′	−
$MycB_u$	5′-GCTCAGATCTCCATGGAGCAAAAGCTCATTTCTGAAGA-3′	−
$MycB_1$	5′-ACGGAGATCTAATTCAAGTCCTCTTCAGAAATGAGCTT-5′	−
NEO	5′-GTTCAATCATTGATCAGATC-3′	−
1.3/3′	5′-TGCTCTAGAGCAAGCCGCCGCCCACTTGTG-3′	−
NEO 2	5′-CCGGATCCGGCCCAAGCGGCCGGAGAACCTGC-3′	−
CAL5′ A	5′-CTCTAGACCGCAATCCCGGCCCGTGCG-3′	−
CAL5′ B	5′-CTCTAGAGCCCAAGTGTGAAGGAAAAAGG-3′	−
CAL3′	5′-GGAGTTCCGCCTCGGTGGGG-3′	−
OL9	5′-TAGAACAGTTTCTGTACTATA-3′	−
OL8	5′-ATGGCCGATCAACTCTCCAAC-3′	−
OL7	5′-TTACCCGCAGGACATATCCAC-3′	−
OL6	5′-AATTCCCTCATGACATGTCTT-3′	−
OL5	5′-AGACCTCAGACTGCTCATTTG-3′	−
OL3	5′-GACCCTGTACCTGCAAATGAG-3′	−
OL2	5′-CATGGAGAGACGATTCATCAT-3′	−
Poly(dC)		−
Subtel-C (RNA)	5′-GGGAACA$_4$GCUGGGUACCGGGC$_7$UCGAGGUCGAGGUAUCGAUA AGCUCCCGCUAACCCGAAACACCCUAACCCGAAAGCUUGAU-3′	−
Subtel-G (RNA)	5′-GGGCGAAUUGGAGCUCCACCGCGGUGGCGGCCGCUCUAGAAC UAGUGGAUC$_5$GGGCUGCAGGAAUUCGAUACAAGCUUUCGGGU UAGGGUGUUUCGGGUUAGCGGGAGCUUAU-3′	−

[a] Various single-stranded DNAs and RNAs were tested for inhibition of the double-stranded subtelomere repeat (Subtel-ds) complex. +, competition; −, no competition. The plus sign in parentheses indicates partial inhibition. Underlined portions of sequences are the sequence of the subtelomere repeat.
[b] All competitors except the last two are DNA competitors; Subtel-C (RNA) and Subtel-G (RNA) are RNA competitors.

binding buffer (see above) and 10 mM KCl. Proteins were eluted by either a continuous (0.1 to 2 M KCl) or a step (0.1, 0.5, 1, 1.5, and 2.0 M KCl) gradient.

Peak fractions from the Affi-Gel Blue column (15 ml) were pooled and desalted on a G25-Sephadex column (45 ml) equilibrated with MOPS buffer (20 mM morpholinepropanesulfonic acid [MOPS] [pH 7.0], 0.1 mM EDTA, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol) and 10 mM KCl. The desalted material was then loaded on a Bio-Rex 70 column (10 ml) in MOPS buffer and eluted with a KCl step gradient (0.1, 0.2, 0.3, 0.5, and 0.7 M).

Renaturation of ST-1 was done as described previously (6), except for removal of guanidine chloride, which was done by dialysis instead of with a gel filtration column (37a). One milliliter containing the peak activity from the Bio-Rex column was pooled, trichloroacetic acid precipitated, and electrophoresed on a 12.5% polyacrylamide-sodium dodecyl sulfate (SDS)-protein gel. The gel was then cut in slices which were individually soaked in elution buffer (50 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 0.1% SDS, 5 mM dithiothreitol, 150 mM NaCl, 0.1 mg of bovine serum albumin per ml) overnight. The eluate was then precipitated with 4 volumes of acetone, denatured in 75 μl of 6 M guanidine hydrochloride in binding buffer plus 0.1 M KCl, and then slowly renatured by dialysis against binding buffer plus 10 mM KCl. All protein purification steps were conducted at 4°C.

Pulsed-field gel electrophoresis and DNA transfer. Intact trypanosome DNA was resolved on a pulsed-field 1% agarose gel (46), under conditions designed to separate the three different chromosome size classes (150 V, 150-s pulse, 60 h, 0.5× TBE buffer, and 14°C). The DNA was transferred to nylon membranes (GeneScreen), hybridized to DNA probes (50% formamide-dextran sulfate) and washed, all according to the manufacturer's recommendations.

RESULTS

A binding activity in *T. brucei* has specificity for the telomere repeats but a higher affinity for the 29-bp subtelomere repeats. To isolate *T. brucei* proteins specific for the trypanosome telomere repeats, S-100 cellular extracts from two major developmental forms of the parasite (procyclic and bloodstream) and a double-stranded radiolabeled oligomer which contains three tandem copies of the trypanosome telomere hexamer repeat 5′-CCCTAA-3′ (45), (Tel-ds [Table 1]) were used in binding assays. Tel-ds forms two complexes of retarded gel mobility, and this was observed with numerous extracts of trypanosomes at both stages (Fig. 1A, lanes 2 to 6). At small amounts of extract, the faster-migrating complex is predominant (Fig. 1A), but with larger amounts of extract relatively more of the upper complex was observed (compare Fig. 1A and B). A DNA oligomer carrying four rather than three copies of the repeat gave the same pattern of complexes (data not shown). This complex formation is also sequence specific. It was inhibited by an excess of unlabeled Tel-ds oligomer, but the complex intensity was not diminished by the addition of any of seven examined nonspecific double-stranded DNAs. These nonspecific DNAs include R-Tel-ds, a rearranged repetitive oligomer analogous to Tel-ds but in which the second and fifth residues of each telomere repeat are exchanged, thus maintaining the nucleotide composition and G- and C-strand bias of Tel-ds (Fig. 1B, Table 1, and data not shown).

Formation of the gel-retarded complexes on the telomeric repeats was also resistant to high salt concentrations up to 2 M KCl (for an example, see Fig. 1B), indicating a tight binding. To rule out the unlikely possibility that all the binding observed in Fig. 1B occurred only after the sample began running into the gel, we performed control experiments in which an excess of unlabeled Tel-ds competitor was added just before the gel was loaded. This did not alter the results from those shown in Fig. 1B, confirming that the binding indeed occurred under high-salt conditions (data not shown).

Abutting the telomere repeats in numerous *T. brucei* chromosomes are 29-bp repeats made of telomere-derived sequences (49). We next assayed the *T. brucei* S-100 extracts for binding to these subtelomere repeats by using radiolabeled

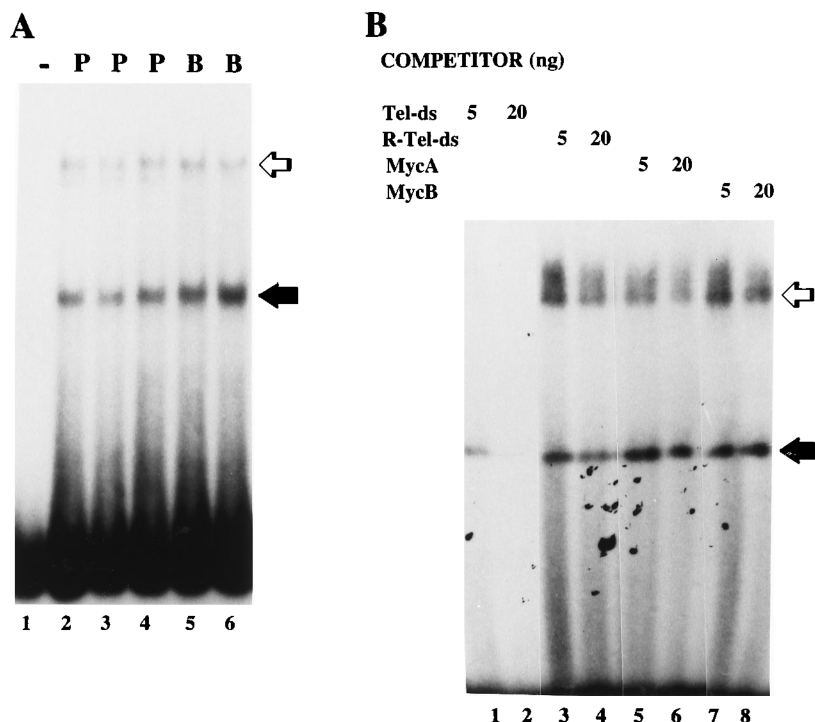


FIG. 1. Telomere-specific binding activity in *T. brucei*. (A) Twenty femtomoles (0.35 ng) of radiolabeled Tel-ds oligomer (Table 1) was incubated with no extract (−) or with 0.2 μg of three different procyclic-form (P) or two different bloodstream-form (B) *T. brucei* S-100 extracts for 20 min and was resolved on a 4% polyacrylamide gel. (B) Competition assays done at 1.5 M KCl (analogous results were obtained at 100 mM KCl). Twenty femtomoles (0.35 ng) of radiolabeled Tel-ds was incubated with 5 μg of procyclic-*T. brucei* extract that had been preincubated in the presence of the indicated amount (in nanograms) of the same (lanes 1 and 2) or nonspecific (lanes 3 to 8) (Table 1) unlabeled competitors. Complexes without competitor look like those formed with the nonspecific competitors. R-Tel-ds has the same nucleotide composition as Tel-ds, but the nucleotides were arranged in a random order (Table 1). The heavy solid arrow indicates the complex examined in this study, and the open arrow indicates the more-slowly-migrating complex that involves a different extract component (Fig. 3B).

Subtel-ds, an oligomer containing one such 29-mer box (Table 1). Under the same conditions used for binding to Tel-ds, Subtel-ds formed three complexes of retarded gel mobility, and again these were formed with several different extracts from both procyclic-form and bloodstream form trypanosomes (Fig. 2A, lanes 2 to 5). Formation of the faster-migrating and predominant complex was also salt resistant, up to at least 2 M KCl (data for these and control experiments not shown). Inhibition of this complex formation by unlabeled Subtel-ds, but not by five examined nonspecific double-stranded DNAs, in-

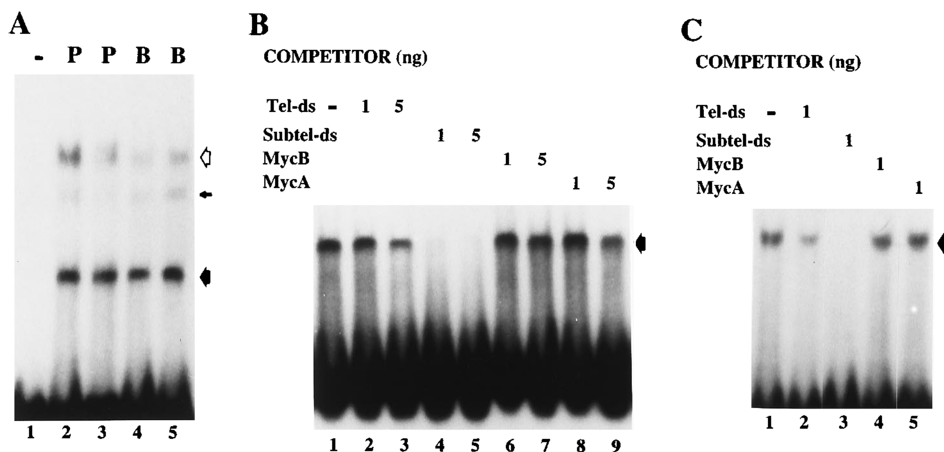
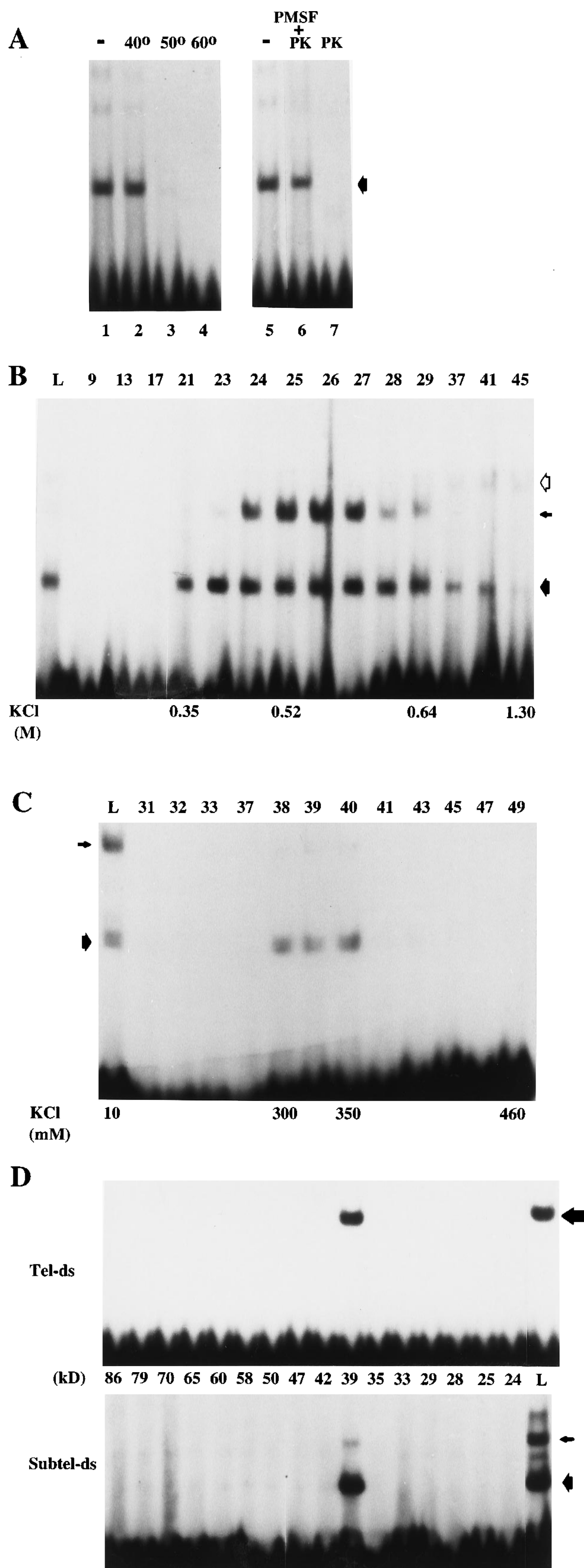


FIG. 2. Subtelomere-specific binding activity in *T. brucei*. (A) Twenty femtomoles (0.50 ng) of radiolabeled Subtel-ds (Table 1) was incubated with no extract (−) or with 0.2 μg of two different procyclic-form (P) or two different bloodstream form (B) *T. brucei* S-100 extracts as for Fig. 1. (B) Competition assays were performed with 20 fmol (0.50 ng) of radiolabeled Subtel-ds oligomer and 0.2 μg of protein extract which had been preincubated with no competitor (lane 1) or the indicated amount (in nanograms) of the same (lanes 4 and 5) or other (lanes 6 to 9) unlabeled competitor oligonucleotides. When larger amounts of extracts are used (e.g., conditions as for Fig. 1B), larger amounts of competitors are needed. (C) Twenty femtomoles (0.35 ng) of radiolabeled Tel-ds was incubated with 0.2 μg of an extract that had been preincubated with no competitor (lane 1) or various competitor oligonucleotides at a concentration (1 ng) that was lower than needed for the specific oligomer Tel-ds to fully inhibit in this extract. Heavy solid and open arrows, same as in Fig. 1; thin solid arrow, complex of intermediate mobility (see Results and Fig. 3).



cluding R-Tel-ds, demonstrated its sequence specificity (Fig. 2B and Table 1).

We next wanted to determine whether the complexes that form on Tel-ds and Subtel-ds use the same or distinct activities of the trypanosome extracts. To this end, cross-competition experiments were performed. The telomere repeat (Tel-ds), when used unlabeled and in excess, did not efficiently inhibit formation of the complex on Subtel-ds (Fig. 2B, lanes 2 and 3). A limited extent of inhibition of complex formation on Subtel-ds may be observed at higher concentrations of Tel-ds (Fig. 1B, lane 3), but it requires much more of the Tel-ds competitor than is needed to equivalently inhibit binding to the Tel-ds (Fig. 2B, lanes 2 and 3; compare with Fig. 2C, lane 2). However, when the experiment was performed in reverse, with a radiolabeled Tel-ds probe, the unlabeled Subtel-ds oligomer fully inhibited complex formation on the radiolabeled Tel-ds (Fig. 2C, lane 3). In fact, it did so considerably more efficiently than did the unlabeled Tel-ds oligomer, since inhibition was complete with a lower concentration of the competitor oligomer (Fig. 2C, lanes 1 to 3; compare with Fig. 1B, lanes 1 and 2, and Fig. 2B, lanes 2 to 5). These inhibition results indicate that complex formation on the telomere and subtelomere repeats involves a common trypanosome binding activity and that this activity has a higher affinity for the subtelomere over the telomere repeats. This was a striking finding, since this is the first reported activity that specifically binds both the subtelomere and the telomere repeats of the organism in which it is found.

In further experiments, a DNA fragment containing 12 copies of the telomere repeat (pTel 12; Table 1) showed a limited inhibition of the formation of the Subtel-ds complex, but the inhibition was still not nearly as efficient as that by the Subtel-ds oligomer (data not shown). This confirms that the stronger inhibition of the complex formed by Subtel-ds is not due to a larger potential number of binding sites, since Subtel-ds contains the equivalent of 4 imperfect telomere repeats while Tel-ds and pTel 12 consist of 3 and 12 (canonical) telomere repeats, respectively.

The telomere- and subtelomere-binding activity is a 39-kDa protein, ST-1. Because of its higher affinity for the subtelomere

FIG. 3. Telomere- and subtelomere-repeat-binding activity is a 39-kDa polypeptide. (A) S-100 extracts were used untreated (lanes 1 and 5) or were heated for 10 min at the indicated temperatures and then pelleted in the cold, and the supernatant was assayed (lanes 2 to 4), or the extracts were digested with proteinase K (PK) (0.5 μg/10 μl of extract for 20 min at room temperature) in the presence or absence of 10 mM phenylmethylsulfonyl fluoride (PMSF) (lanes 6 and 7), before binding to 20 fmol (0.50 ng) of radiolabeled Subtel-ds. (B) S-100 extract was fractionated by Affi-Gel Blue column chromatography as described in Materials and Methods, and 1 μl of each fraction (indicated by number at the top) was assayed for binding to 20 fmol (0.50 ng) of radiolabeled Subtel-ds. The polypeptide that causes the rapidly migrating complex (thick solid arrow) was investigated in this study; a complex of intermediate migration that became prominent with high concentrations of the peak fractions (thin solid arrow) is due to more than one copy of this same polypeptide (see panel D). The complex designated with an open arrow is due to a factor(s) that elutes at higher salt concentrations and was further studied but not described in this paper. Lane L shows the S-100 extract that was loaded on the column. (C) The peak Affi-Gel Blue fractions (21 to 29) were further resolved by Bio-Rex 70 column chromatography. Five microliters of each fraction (indicated by number at the top) was assayed for binding to the Subtel-ds oligomer. Lane L contains the loaded Affi-Gel Blue material. (D) The peak Bio-Rex fractions (38 to 40) were trichloroacetic acid precipitated and resolved by electrophoresis on a 12.5% polyacrylamide-SDS-protein gel. The entire lane was cut into slices, using as standards the Bio-Rad high-molecular-mass markers (200 to 14 kDa). After elution and renaturation with guanidine chloride, the proteins corresponding to the designated molecular masses were tested for binding to both the telomere (top of panel) and the subtelomere (bottom of panel) repeats. Lane L is the loaded Bio-Rex material. The arrows are as defined for panel B and Fig. 1.

repeat, we characterized the subtelomere- and telomere-binding activity with Subtel-ds as the substrate oligomer. The component of the *T. brucei* extract that forms this complex was sensitive to heat treatment (Fig. 3A, lanes 3 and 4). It was also sensitive to proteinase K digestion (Fig. 3A, lanes 5 to 7). However, it was resistant to treatment with several different nucleases (RNase A, RNase T_1, and micrococcal nuclease; data not shown). This indicates that the binding activity in the trypanosome extract is a protein and that it does not have a detectable RNA component.

To purify the binding activity, S-100 extracts were fractionated on Affi-Gel Blue columns. The subtelomere-binding activity eluted between 350 and 650 mM KCl (Fig. 3B). We also observed a complex of intermediate mobility peaking in the same salt range. When smaller amounts of the fractions are assayed, this complex becomes much less abundant than the faster-migrating one (data not shown). Because of this concentration dependence and because this complex is also observed with the gel-purified renatured protein (Fig. 3D, bottom), the complex is most likely due to more than one copy of the subtelomere-binding protein associated with a single DNA molecule. Also shown in Fig. 3B is a slowly migrating complex that eluted at a higher salt concentration and corresponds to the upper complex in Fig. 2.

The fractions with peak subtelomere-binding activity from the Affi-Gel Blue column were pooled and further resolved on a Bio-Rex 70 column. The binding activity was again recovered as a single peak, eluting around 300 mM KCl (Fig. 3C). Between 30 and 40% of the total binding activity was recovered after the two chromatography steps, during which it was purified up to 500-fold.

The active Bio-Rex 70 fractions were then pooled, concentrated, and resolved on an SDS-protein gel. Slices of the gel were eluted, renatured, and assayed for binding activity. One protein band, with an apparent molecular mass of 39 kDa, regenerated both the Subtel-ds and the Tel-ds binding complexes (Fig. 3D); these complexes comigrate with those formed by the proteins loaded onto the SDS gel (Fig. 3D, lane L). None of the remaining slices in the entire lane yielded proteins that bound to either of these DNAs by gel shift analysis (Fig. 3D and data not shown). Renaturation results identical to those in Fig. 3D were obtained whether the SDS gel was loaded with Affi-Gel and Bio-Rex-purified material, with Affi-Gel Blue-enriched material, or with total extract.

The renaturation data confirmed that the Tel-ds and Subtel-ds complex-forming activity is the same polypeptide of 39 kDa. We named it ST-1 for subtelomere- and telomere-binding activity. Moreover, the reappearance of the complex described above as having intermediate migration and forming in a highly protein concentration-dependent manner on Subtel-ds (Fig. 3B) when the renatured protein band of ST-1 was used (Fig. 3D) provides further evidence that it is due to more than one copy of ST-1 binding to Subtel-ds and that it does not involve a polypeptide of a different size.

ST-1 binds the C-rich strand of the 29-bp subtelomere repeat. To compare ST-1 with the telomeric-interactive proteins identified to date from various species, ST-1 activity was next tested for affinity for various single-stranded nucleic acids. Strikingly, the C-rich strand of the 29-bp subtelomere repeat (Subtel-C) was able to efficiently inhibit the ST-1 complex on Subtel-ds (Fig. 4A and B, lanes 3). Of the 27 competitor single-stranded DNAs used in this study (Table 2), only the Subtel C-rich strand was able to efficiently inhibit the binding of Subtel-ds. Its complementary G-rich strand (Subtel-G), poly (dC), and the 24 other single-strand oligonucleotides we tested failed to do so (Fig. 4A and B and data not shown). These nonspecific competitors include NEO 2 and CAL5′ A, which are comparably C rich and have blocks of C residues similar to those of Subtel-C. The in vitro-synthesized RNA versions of both Subtel-C and Subtel-G also did not inhibit complex formation on Subtel-ds (Table 2; Fig. 4B, lanes 6 and 7; and data not shown). As with the double-stranded oligomers (Fig. 2B and C), only the telomere C-rich strand (Tel-C) may exhibit a limited inhibition, but at considerably higher concentrations of DNA than are needed for inhibition by Subtel-C (Fig. 4B and data not shown). Control experiments (data not shown) demonstrated that the inhibition by Subtel-C is not due to its displacing and liberating the C-rich strand of the radiolabeled Subtel-ds duplex in the binding reaction, because then we would have observed the liberated C-rich strand, which migrates faster than Subtel-ds, yet this was not observed. Furthermore, competitor titrations showed that the affinity of ST-1

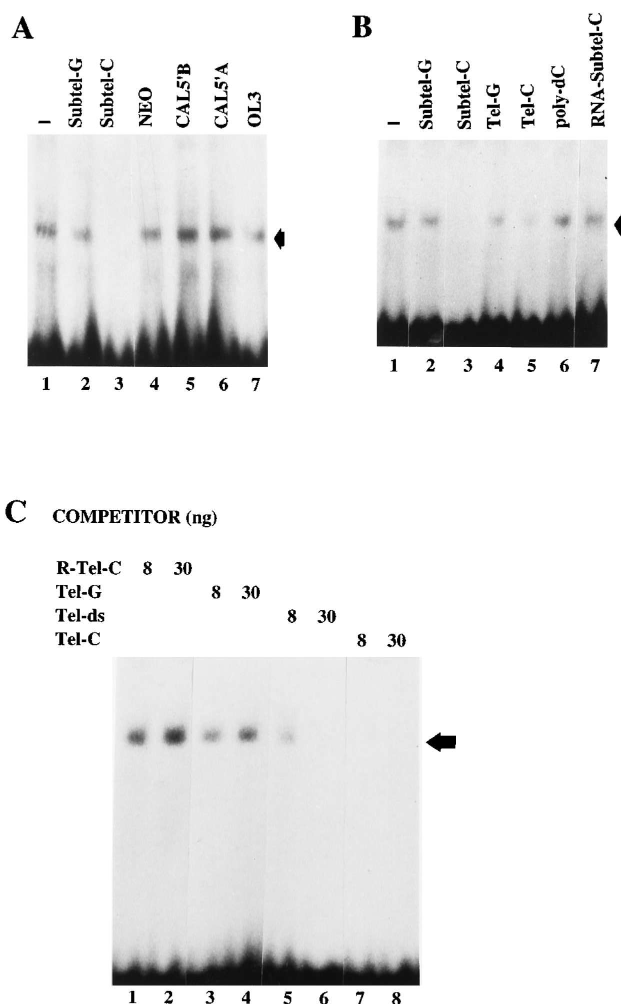


FIG. 4. ST-1 binds with high affinity to the subtelomere C-rich strand. (A and B) Competition assays performed with 20 fmol (0.50 ng) of radiolabeled Subtel-ds oligomer and extract (0.2 μg of extract protein) that had been preincubated in the absence (lanes 1) or presence of 1 ng of various single-stranded DNA or RNA unlabeled competitors, as designated (Table 2). Subtel-G and Subtel-C and Tel-G and Tel-C are the homologous single strands of Subtel-ds and Tel-ds, respectively. RNA-Subtel-C is the in vitro-synthesized transcript corresponding to Subtel-C. (C) Competition assays performed with 20 fmol (0.35 ng) of radiolabeled Tel-ds and 5 μg of S-100 extract that had been preincubated with the indicated amount (in nanograms) of the designated unlabeled competitors. R-Tel-C has a nucleotide composition identical to that of Tel-C but with the nucleotides arranged in a random order.

for the C-strand of the subtelomere repeat (Subtel-C) was comparable to that for its double-stranded form (Subtel-ds; data not shown). These results have also been confirmed in analogous competition binding analyses in which the single-stranded Subtel-C was the radiolabeled substrate and various double- and single-stranded oligomers were used as unlabeled competitors (data not shown).

In competition assays with Tel-ds as the radiolabeled substrate (Fig. 4C), ST-1 also showed an affinity for the C-rich strand of the telomere repeat (Tel-C [Table 2]). It did not show comparable binding to the complementary G-rich strand (Tel-G), to R-Tel-C, or to the four Myc oligomers examined (Fig. 4C, Table 2, and data not shown). As with the subtelomere repeats, titration competition experiments revealed that the affinity of ST-1 for Tel-C was about the same as for Tel-ds; however, it was about 10-fold lower than its affinity for Subtel-C or Subtel-ds (data not shown). Thus, the order of preference of binding of ST-1 is as follows: nonspecific sequence $\ll$Tel-ds ~ Tel-C < Subtel-ds ~ Subtel-C. ST-1 does not bind RNA or numerous other oligomers, even ones that are as C rich as the Tel-C and Subtel-C strands and/or have runs of C residues similar to those of the Tel-C and Subtel-C strands.

Examination of the DNA in the complex formed by ST-1 with input subtelomere and telomere duplex DNA revealed the presence of both the C-rich and the G-rich strands at equal levels (data not shown). In this experiment, duplex DNA was formed with only the C-rich strand labeled, only the G-rich strand labeled, or both strands labeled. In all cases, a gel-retarded radiolabeled complex of the same migration was formed with equal efficiency, showing that both the C- and the G-rich strands are present in the gel-retarded complex that forms on the double-stranded input DNA. While this demonstrates that the binding of ST-1 does not result in the complete denaturation of these duplex DNAs, it does not rule out the possibility that ST-1 binding perturbs their structure.

Distribution of the 29-bp subtelomere repeats in the trypanosome genome. When resolved on a pulsed-field gel, *T. brucei* chromosomes are classified into three size classes (large, middle, and mini chromosomes [46]). Chromosomes of all these size classes carry the 29-bp subterminal repeats (Fig. 5, right lane). This particular pulsed-field gel was run to resolve the middle-sized chromosomes. Unlike the telomere repeats which were present on every chromosome (Fig. 5, left lane; ethidium bromide staining for total DNA yields the same band pattern as probing for Tel sequences [data not shown]), the subtelomere repeats were not present on every chromosome (Fig. 5, right lane); only two of the five middle-size chromosomes had them. This distribution is consistent with that of repetitive elements reported for the subtelomeric regions of other organisms (see Discussion).

Digestion from the ends of large chromosomal DNA with *Bal* 31 nuclease and selective hybridization analysis (data not shown) showed that most of the subtelomere repeats in *T. brucei* had a sensitivity intermediate between those of the more rapidly digested telomere repeats and the relatively insensitive bulk DNA typified by the tubulin gene. This corroborates prior data showing that the 29-bp repeat sequences are near the ends of chromosomes but centromeric to the telomere repeats (45). Quantitation of repeats in genomic Southern hybridizations with subcloned subtelomere and telomere probes showed ~7,000–10,000 subtelomere repeats per genome. The number of the telomere repeats was determined to be 50-fold higher than that of the subtelomere repeats, confirming previous data (45).

By dilutional quantitation of the gel shift analysis, ST-1 was seen to be present at a few thousand copies per cell equivalent of the S-100 extract (data not shown). An approximately equal abundance was observed in Dignam nuclear extracts (data not shown). If about half of the ST-1 in the cell was recovered, this suggests that there may be one ST-1 protein molecule bound to each subtelomere repeat. Given the resistance to high salt concentrations of the ST-1–Subtel-ds complex, however, it is reasonable to assume that an appreciable fraction of cellular ST-1 might remain bound to genomic DNA and not be recovered in the cell extracts. Nonetheless, SDS gel analysis of whole *T. brucei* cells indicates that there are considerably fewer ST-1 molecules than would be present if there was one bound to each telomere repeat in the chromosomes.

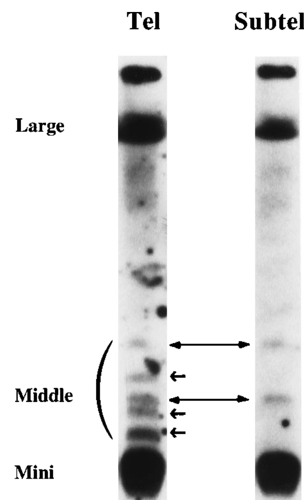


FIG. 5. Presence of telomere and subtelomere repeats on *T. brucei* chromosomes. A Southern transfer of intact trypanosome chromosomal DNA was run on a pulsed-field gel under electrophoresis conditions which separate the three distinct size groups, large chromosomes (top), medium chromosomes (resolved in center), and minichromosomes (bottom). Radiolabeled Tel-ds and Subtel-ds were used as probes to visualize the telomeres (Tel) and the subtelomere repeats (Subtel). Ethidium bromide staining showed that all chromosomes are visualized with the telomere probe (single and double-headed arrows) while a subset are visualized with the subtelomere probe (double-headed arrows).

DISCUSSION

In this study we identified ST-1, a 39-kDa protein (Fig. 3) in *T. brucei* with unique binding properties. ST-1 is the major telomere-binding activity in the parasite extracts (Fig. 1A). In addition to its binding specificity for the duplex form of the canonical trypanosome telomere repeats of CCCTAA (Fig. 1B), ST-1 bound at least 10-fold more strongly to the 29-bp subtelomere repeats (Fig. 2), which consist of perfect and imperfect telomere repeats (49) located immediately proximal to the simple telomere DNA. Proteins that bind to both their homologous subtelomere and telomere repeats have not previously been described for other organisms. In fact, the only other protein known to bind a telomere-degenerate subtelomere sequence is TBFα (32), an essential protein in *Saccharomyces cerevisiae* (5).

The 29-bp elements in *T. brucei* (49), like the 27-bp stretches in *P. berghei* (37) and equivalent segments in many other eukaryotes, are found at the border separating the terminal telomere block from the more complex and frequently rearranging subtelomeric region. This fluid (41) region at the subtelomeric ends of chromosomes is distinguished by the presence of nonconserved middle-repetitive elements called TASs (29, 38, 41). TASs are thought to be regions of high-frequency recombina-

tion events responsible for DNA exchange between nonhomologous chromosomes (references 17 and 50 and references therein), meiotic pairing and recombination (42), chromosome size polymorphisms (12), and amplification (reference 33 and references therein). As can be seen by pulsed-field gel electrophoresis data (Fig. 5), many but not all *T. brucei* chromosomes carry the 29-mer repeats, a distribution consistent with the general organization of telomere-variant repeats and TASs in other eukaryotes (10). TASs in trypanosomes are AT- or GC-rich boxes that are present on most chromosomes (49), just centromeric to the 29-bp subtelomere repeats, and in at least one instance separating the VSG gene from the telomere repeats (16). A frequently noted and important example of subtelomeric rearrangement events is the conversion and diversification of the antigen genes (VSGs) in *T. brucei* (4).

The position of the 29-bp repeats at the border between the telomere repeats and the TASs may implicate such elements as hinge regions delineating the recombination domains at chromosome ends. Indeed, the 27-bp telomere-derived elements in *P. berghei* formed the proximal and distal junctions of the 2.3-kb subtelomeric element insertion events which resulted in chromosome size polymorphisms in these parasites (40). The apparent paucity of ST-1 compared with the number of telomere repeats (see Results), combined with its much greater in vitro affinity for the 29-bp subtelomeric sequence than for the telomere repeats (Fig. 2), could mean that in vivo, ST-1 binds the subtelomeric 29-mer DNA preferentially over the telomeres. ST-1 could therefore have a function different from those of known telomeric proteins thought to either coat the telomere double-stranded repeats (53) or cap the chromosome terminus (26). If the subtelomeres are indeed hinge regions at the edges of recombination domains, it makes ST-1 a possible player in rearrangements of the trypanosome VSG. Evidence for an effect of telomere region interactions on VSG gene conversion underlying antigenic variation was suggested on the basis of altered activation of a VSG gene that was present in the opposite orientation with respect to the telomere repeats (47). Our data show that ST-1 is present in both bloodstream and procyclic trypanosomes (Fig. 1A and 2A); it should be noted that the genome of procyclic as well as bloodstream trypanosomes is known to be plastic and to relatively frequently rearrange (21).

In addition to its affinity for the double-stranded subtelomere as well as telomere repeats, ST-1 also shows affinity for the single-stranded versions of these sequences. Unlike all other previously described telomere-binding proteins, however, whose single-strand affinity is solely for the protruding G-rich strand of the repeat, the single-strand affinity of ST-1 is for the C-rich strand of the telomere repeat (Fig. 4C) and, about 10-fold greater, for the C-rich strand of the 29-bp subterminal repeats (Fig. 4A and B). No analogous affinity was seen for a large number of other single-stranded DNAs, including ones that are as C rich as the subtelomere and telomere C-rich strands and have blocks of C residues similar to those of the subtelomere and telomere C-rich strands (Table 2). This unprecedented affinity for the C-rich strand may reflect the structure of the trypanosome subtelomeres and the mechanism of ST-1 binding to them. While our data show that ST-1 does not fully denature the duplex telomere and subtelomere repeats upon binding, they do not exclude the possibility that DNA bound in the ST-1 complex may be in a partially single-stranded state.

In *S. cerevisiae*, the boundary between the nonnucleosomal telomeric DNA and the adjacent nucleosomal subtelomeric Y′ elements was hypersensitive to DNase I treatment and accessible to *Escherichia coli dam* methylase, suggesting that it may be a transition region between two different chromatin structures (51). By analogy, the 29-bp subtelomere repeats at the trypanosome telomeres could also have an altered structure, possibly with a single-stranded character, consistent with the affinity of ST-1 for the C strand of the repeats. One could hypothesize that in the cell, ST-1 binds the C strand of the repeat already opened by other proteins or that it recognizes the subtelomeric duplex, either helping to unwind it or making contact with the C strand alone while in the duplex form. ST-1 evidently does not have two independent and nonoverlapping domains for binding the single-stranded and double-stranded forms, or for binding the telomere and subtelomere repeats, since each competes with the other in the gel shift analysis. The combination also does not show an altered shift position that would be indicative of two oligomers binding to one protein molecule.

The binding specificity of ST-1 both for the duplex form of the recognition sequence and for one strand of this sequence is a property shared by a special group of gene regulators, like the α1 and MCM1 factors in *S. cerevisiae* (25), the ovalbumin gene complex (36), the estrogen receptor (31), and the mammary tumor virus repressor in mammalian cells (22). This dual affinity of binding may be a general feature that extends beyond the realm of transcription effectors. The only other reported protein from a trypanosomatid with affinity for telomeric sequences is one from *Crithidia fasciculata* (44) which binds the G-rich strand of the telomere repeats but has a 10-fold-greater affinity for the mitochondrial replication origin.

The fascinating events of telomeres in general, and those of *T. brucei* in particular, make the unprecedented binding properties of ST-1 most interesting. Study of ST-1 function could help elucidate the nucleoprotein organization and geometry at *T. brucei* chromosome ends and their possible effect on telomere-associated gene expression, rearrangement, and nucleotide modification (4, 23).

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