A Trypanosoma brucei Protein Complex That Binds G-overhangs and Co-purifies with Telomerase Activity*

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Maria Isabel N. Cano¶¶¶, Julie Johnson Blake¶, Elizabeth H. Blackburn§, and Nina Agabian¶¶**

From the Departments of ¶Stomatology, ¶¶Microbiology and Immunology, and **Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0422

The chromosomal ends of Trypanosoma brucei, like those of most eukaryotes, contain conserved 5'-TTAGGG-3' repeated sequences and are maintained by the action of telomerase. Fractionated T. brucei cell extracts with telomerase activity were used as a source of potential regulatory factors or telomerase-associated components that might interact with T. brucei telomeres. Electrophoretic mobility shift assays and UV cross-linking were used to detect possible single-stranded telomeric protein-DNA complexes and to estimate the approximate size of the protein constituents. Three single-stranded telomeric protein-DNA complexes were observed. Complex C3 was highly specific for the G-strand telomeric repeat sequence and shares biochemical characteristics with G-rich, single-stranded telomeric binding proteins and with components of the telomerase holoenzyme described in yeast, ciliates, and humans. Susceptibility to RNase A or chemical nuclease (hydroxyl radical) pre-treatment showed that complex C3 was tightly associated with an RNA component. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry was used to estimate the molecular mass of the peptides obtained by in-gel Lys-C digestion of low abundance C3-associated proteins. The molecular masses of the peptides showed no homologies with other proteins from trypanosomes or with any protein in the data bases screened.

Telomeres are specialized protein-DNA complexes that form the physical ends of eukaryotic chromosome (1). In Trypanosoma brucei, as in other eukaryotes, the essential telomeric DNA consists of duplex tandemly repeated sequences of 5'-TTAGGG-3' (2, 3), which protrude toward the end of the chromosomes as 3' G-rich overhangs that eventually form t-loop structures (4, 5). The lengths of double-stranded telomeric DNA and of distal, G-rich single-stranded DNA vary considerably between organisms and during the cell cycle, as well as between different chromosomes in the same organism (6–9).

The higher order protein-DNA complex that caps the end of the chromosomes is composed of a number of proteins that are associated with the double-stranded telomeric DNA, with the single-stranded G-rich overhang, and with other accessory proteins (10–14). The abundant yeast protein Rap1p, for example, binds to the double-stranded telomere and to the most distal telomere sequences at the chromosome terminus, where it acts as a negative regulator of telomerase elongation (13, 15). Rap1p also interacts with the telomeric silencing information regulator proteins and controls the expression of genes positioned in the proximity of telomeres (14, 16, 17). Proteins that share the same functions with the yeast Rap1p and a mammalian Rap1p ortholog have also been described (18, 19).

Telomerase reverse transcriptase, the enzyme that replicates eukaryotic telomeres, is one of the better-characterized factors that bind specifically to the 3'-G-rich overhang DNA. In addition to telomerase, some non-conserved single-stranded telomeric proteins that associate with the G-rich strand co-purify with telomerase activity or the telomerase core subunits. The p80p95 complex of Tetrahymena thermophila and the yeast Est1p interact with the telomerase RNA component and are physically associated with telomerase activity in vivo (10, 12, 20). Other proteins, such as the yeast Cdc13p (21) and the αβ complex of Oxytricha nova (22), are indirectly associated with components of the telomerase holoenzyme and act synergistically with other molecular components of the telomeric complex (23). Genetics and biochemical experiments in both yeast and mammals have shown that these telomere-protein interactions (10–12, 20, 23) are mainly responsible for (i) regulating telomerase activity (23, 24), (ii) capping the telomere ends to protect them from nuclease digestion or chromosome end-to-end fusion (24–29), (iii) promoting chromosome pairing during mitosis and meiosis (18, 30), (iv) controlling expression of genes positioned at the telomeres (14, 31), and (v) healing the chromosomes during events of genome rearrangement (32, 33) and thus are essential for maintaining genome stability.

In the African trypanosome T. brucei, the etiologic agent of sleeping sickness, the telomeric environment is necessary (but not sufficient) for the expression of variant surface glycoprotein genes or antigenic variation (34, 35). Antigenic variation is essential for parasite survival in its mammalian host and occurs when one of thousands of different variant surface glycoprotein genes becomes transcriptionally active. Transcriptional activation involves a complex and only partially understood set of genomic rearrangements or gene conversion events that place the variant surface glycoprotein gene in question in a permissive telomeric environment (34–36). In related parasites, such as Leishmania sp. and Trypanosoma cruzi, telomeres and telomere-associated sequences are frequent sites of recombination/amplification events (5, 33, 37, 38), which may contribute to genomic variability/plasticity.

Eid and Sollner-Webb (39, 40) described two T. brucei telomeric proteins, St1p and St2p, which are detected in S100 extracts of procyclic and bloodstream forms of the parasite.
Each protein participates in the formation of two major telomeric complexes. The 39-kDa St1 protein preferentially binds to the double-stranded 29-bp subtelomeric repeat region and to double-stranded telomeric repeats, exhibiting greater affinity for their C-rich single-strands (39). St2 is a complex of proteins with affinity for duplex telomeric and subtelomeric DNA sequences and has a greater affinity for the G-rich strand of the telomere repeat. However, the greater affinity of this complex is for the G-rich strand of the 29-bp subtelomeric repeat. St1p is necessary for St2 DNA binding and is apparently part of the complete St2 DNA binding complex (40). Field and Field (41) also reported the existence of a G-strand-binding protein in T. brucei nuclear extracts, but the protein-DNA complex was not identified or characterized. Recently, Cano et al. (42) described telomerase activity in semipurified extracts of three evolutionarily diverse kinetoplastid protozoa (T. brucei, Leishmania major, and Leishmania tarentolae). Some biochemical properties of the T. brucei telomerase were determined, and a minimal nine-nucleotide sequence for the template region of the T. brucei telomerase RNA component was deduced from enzymatic activity profiles.

In this report, we used telomerase-containing fractions from T. brucei extracts as a source of potential components of the telomerase holoenzyme or of telomeric factors that might regulate T. brucei telomere maintenance. We identified three G-rich single-stranded telomeric complexes (C1, C2, and C3). The proteins associated with complex C3 (25- and 18-kDa protein bands) were purified and subjected to MALDI-TOF MS analysis and did not show homology with any other trypanosome or proteins in the data bases searched. We propose that C3-associated proteins are novel T. brucei single-stranded telomeric proteins.

**EXPERIMENTAL PROCEDURES**

### Parasite Strains and Culture Conditions

Procylic forms of T. brucei strain IsTat1.1 were cultivated in Bienen’s synthetic medium (43) supplemented with 5% heat-inactivated fetal bovine serum at 28 °C for 60 h.

### Preparation of T. brucei Extracts

Telomerase-containing fractions were prepared from T. brucei extracts in the presence of protease inhibitors (see Ref. 42 for details). Briefly, S100 fractions were fractionated by DEAE-agarose column chromatography (Biogel; Bio-Rad). The columns were equilibrated with 1× TMG (50 mM Tris-HCl, pH 8.0, 1.5 mM MgCl₂, 10% glycerol) and wash with six volumes of 1× TMG containing 0.3 M sodium acetate, pH 8.0. Activity was eluted in one column volume each of 0.5 M sodium acetate, pH 8.0, in 1× TMG. Fractions were then desalted with Microcon-30 filters (Amicon) to a final salt concentration of 100 mM.

### Telomerase Activity

1 µg of S100 or 1 µg of each DEAE-agarose fraction (300–500 mM sodium acetate) was assayed for telomerase activity using a modification of the two-tube telomere repeat amplification protocol (TRAP) assay as described in Ref. 42. Protein extracts were assayed in a mixture containing 40 pmol of TS forward primer (5′-AACCTGCTCGAG-CAAAGTT-3′), 1× modified TRAP buffer (50 mM Tris-HCl, pH 8.3, 1 mM dithiothreitol, 1 mM spermidine, 1 mM MgCl₂, 2 mM dATP, 2 mM TTP, and 10 µM dGTP at 28 °C for 1 h. 10 µl of this reaction were added to a 50-µl PCR mix containing 50 µl of each non-radiolabeled dNTP, 20 pmol TS primer, 20 pmol CX-ext reverse primer (5′-GTGGCCTTACCCCTTACCCCTTACCATGGAC-3′), and 0.1 µCi of [α-32P]dGTP/µl and 1 unit of Taq polymerase (Invitrogen). The PCR conditions were as described in Ref. 42. The products were fractionated on 10% sequencing gels and autoradiographed. Activity in the 500 mM fraction was tested by pre-treating the extracts with 100 ng of RNase A (Sigma) for 5 min at 37 °C.

### Preparation of Single-stranded, G-overhang, and Double-stranded Oligomers

DNA or RNA oligonucleotides (see Table I for oligonucleotide sequences) were purchased from Operon Technologies and gel-purified before and after 5′ end-labeling with [γ-32P]ATP and T4 polynucleotide kinase (44). Oligonucleotides OvhR and tel C, used to prepare the partial duplex and double-stranded DNAs, respectively, were radiolabeled with [γ-32P]ATP and T4 polynucleotide kinase prior to mixing with the complementary OvhH and tel C oligos. Partial duplex G-rich overhang DNA was obtained by mixing equimolar amounts of radiolabeled OvhR oligonucleotide followed by heat at 95 °C and slow cooling at room temperature (10). The partial annealed duplex containing a 3′ overhang of 17 mer was separated from the residual single-stranded species by purification on a 10% native acrylamide gel and quantified in a β-counter (10). The double-stranded telomeric DNA oligonucleotide was also formed after heating the radiolabeled oligonucleotide tel 3 and non-radiolabeled oligonucleotide tel C at 95 °C, followed by slow cooling at room temperature (40). As tel C and tel 3 were not completely complementary, the double-stranded species formed with this mixture were end-filled with Klenow DNA polymerase and non-radioactive nucleotide triphosphates (40). The fully double-stranded DNAs were purified from the residual single-stranded DNA and quantified as above.

### Electrophoretic Mobility Shift Assay (EMSA)

The S100 and telomerase-positive cell fractions (1 µg each) were incubated individually with 28 pmol of purified 5′-[γ-32P]ATP end-labeled oligonucleotide in a 20-µl reaction containing 25 mM HEPES, pH 7.5, 5 mM MgCl₂, 0.1 mM EDTA, 100 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, and 100 ng of poly(dIdC). Samples were incubated on ice for 30 min before loading onto a 6% native polyacrylamide gel (37.5:1, acrylamide:bisacrylamide, w/w) in 0.5× TBE (0.05× Tris borate and 0.5 mM EDTA, pH 8.3) at 4 °C followed by electrophoresis at 150 V for 3 h. For autoradiography, wet gels were exposed to film for 1 h. Quantitative analysis of the protein:DNA complexes of two independent EMSA was done using the Scion Image processing and analysis program for the IBM PC (www.scioncorp.com). The autoradiographs were scanned and saved in TIFF format, and the corresponding images were calibrated before proceeding with the analysis. The results were plotted as shown in Figs. 1, 2, 3, and 5. The percentage of binding activity of a shifted complex represents the ratio of the density area (arbitrary scanning units) of each shifted complex and the sum of the density areas of all shifted complexes (including unbound oligo) in each lane, multiplied by 100.

### Competition Assays

Non-radiolabeled oligonucleotide competitors were added to the binding reaction at concentrations 10 (0.28 pmol), 50 (1.4 pmol), and 100 times (2.8 pmol) greater than the amount of 5′-[γ-32P]ATP end-labeled oligonucleotides. The influence of the order of addition of competitors relative to the probe was compared by adding the competitors (i) before the probe, followed by 20 min of incubation on ice, (ii) at the same time as the probe, or (iii) after the probe, followed by 20 min of incubation on ice. Preliminary experiments showed no difference in the binding activity between these three orders of addition. The competition assays were therefore done by adding the probe and competitor at the same time.

Quantitative analysis of the shifted complexes in the presence or absence of molar excess of unlabeled competitors was done using Scion Image software as above. The data were normalized to the amount of complex present in the absence of competitor (defined as 100%) and plotted. The Chi-Square test was used to test the null hypothesis of equal distribution of the density areas of complexes C1, C2, and C3 in the presence or absence of unlabeled competitors. We rejected the null hypothesis by a significance level of 0.01 (p < 0.0001). When the expected counts were less than 5, the Fisher’s Exact Test was used.

### Enzymatic and Chemical Treatment of Complexes

**RNase A**—Extract containing 1 µg of protein was incubated with 100 ng of RNase A for 10 min at 37 °C (before or after binding to a labeled oligonucleotide and/or UV cross-linking). In some reactions, 10 units of RNasin/µl was added to the extract just prior to the addition of RNase A and before binding to the oligonucleotide.

**DNase I**—Extract containing 1 µg of protein was incubated for 10...
min at 37 °C, before binding to the oligonucleotide, with 2 units of RQ1 RNase-free DNase I (Promega) in DNase I buffer supplied by the manufacturer. EMSA and UV cross-linking controls were done to confirm that DNase I did not bind to or compete with the probes used or otherwise interfere with the binding reactions (data not shown).

Hydroxyl Radical and Hydrogen Peroxide Treatment—Each of the reagents used for the hydroxyl radical experiments were tested by EMSA. In these experiments, the telomerase extracts (DEAE 500 mM sodium acetate fraction) were pre-incubated with the above reagents prior to the binding reactions and subsequently tested by EMSA. Once shown that the protein-DNA binding was not perturbed by any of the reagents, the extracts were tested in the hydroxyl radical reactions to detect the presence of an RNA component in complex C3. The samples were removed from ice for 1 min and allowed to reach room temperature before hydroxyl radical treatment. Approximately 1 μg of extract was incubated with 2 mM Fe/SO4/m ETDA, pH 8.0, 20 mM sodium ascorbate, and 0.03 or 0.005% H2O2 for 2 min at room temperature. The reaction was stopped by adding 10% glycerol before proceeding with the binding reaction described above. The percentage of binding activity of the complexes after hydrogen peroxide and hydroxyl radical treatment was quantified using the Scion Image software as mentioned before.

Proteinase K Digestion—Extract containing 1 μg of protein was treated with 10 μg of proteinase K (20 mg/ml; Invitrogen) for 10 min at 56 °C before binding.

UV Cross-linking

The 20-μl binding reaction mix was incubated on ice in sealed Eppendorf tubes covered with plastic film and placed 5–7 cm under 254-nm UV lamps for 15 min (45). These irradiation conditions, which produced minimal damage to the telomere oligonucleotide structure and to the complexes, were chosen after exposing free oligonucleotide or the binding reaction mixture to irradiation for different times followed by examination of the UV cross-linked products by SDS-PAGE (data not shown). After irradiation, the samples were mixed with 2× SDS loading buffer (44) for 45 min, boiled for 5 min and loaded onto either 10% or 4–20% pre-cast gradient gels (Bio-Rad). The gels were fixed in 10% methanol, 5% glacial acetic acid for 30 min at room temperature, dried, and exposed for 1–18 h to a Kodak X-Omat film at −80 °C.

MALDI-TOF MS Analysis Sequencing

Approximately 100 μg of the DEAE telomerase fraction (500 mM sodium acetate) was UV cross-linked with 2.8 pmol of unlabeled tel 1 RNA oligonucleotide (as described above) and loaded onto a preparative 20% SDS-PAGE gel containing SDS. UV cross-linking reactions were also done with 5’ end-labeled tel 1 RNA oligonucleotide (see above) and fractions containing telomerase activity, which were run in the same gel containing the unlabeled reagent. The unlabeled bands were excised based on the position of the labeled bands corresponding to the complex C3-associated proteins of 25 and 18 kDa and were immediately frozen in a mixture of dry ice and ethanol. The labeled and unlabeled protein complexes were eluted from the gel matrix, separated by two-dimensional gel electrophoresis at Kendrick Labs, Inc. (www.kendricklabs.com/), and silver stained (unlabeled) or exposed to Kodak X-OMAT film (labeled). The resulting protein spots were in-gel digested with Lys-C (Promega) and subjected to MALDI-TOF MS at the Protein Chemistry Core Facility at Howard Hughes Medical Institute (Columbia University, New York, NY), and to determine the amino acid sequence of the predicted peptide mass, fingerprints were compared with trypsome- some (www.parsun1.path.cam.ac.uk/) and protein data bases (Swiss-Prot) using the Protein Prospector 3.2.1 MS-FIT 3.1.1 analysis program (Baker, P. R. and Clauer, K. R.; www.prospector.ucsf.edu) set at a mass tolerance (accuracy) of 50 ppm.

RESULTS

An RNase A-sensitive T. brucei Complex in Telomerase-containing Fractions Specifically Interacts with Single-stranded G-rich Telomeric DNA—T. brucei S100 extracts and DEAE fractions (300–500 mM sodium acetate) were assayed for the presence of telomerase activity using a modification of the two-tube TRAP assay (Fig. 1A, lanes 2–5). No enzyme activity was detected in S100 (Fig. 1A, lane 1) or in the 300 mM DEAE fraction (Fig. 1A, lane 2). In contrast, the 400 and 500 mM DEAE fractions showed ladders of robust bands characteristic of products elongated by telomerase (Fig. 1A, lanes 3–5). To confirm that the bands were produced by telomerase activity, the DEAE 500 mM fraction was pre-treated with 100 ng of RNase A, which abolished the enzyme activity (Fig. 1A, lane 5).

To identify telomere-binding proteins with the same affinity for the G-rich telomeric strand as telomerase, binding reactions were done using the S100 and DEAE fractions and 5’ end-labeled single-stranded tel 4 oligonucleotide. Three T. brucei telomeric complexes (C1, C2, and C3) were formed and detected at 4 °C using an EMSA in non-denaturing 6% polyacrylamide gels. Two of the three complexes (C1 and C2) were formed using S100 or the DEAE 300–500 mM fractions and oligonucleotide tel 4 (Fig. 1B, lanes 2–6). However, in the 500 mM fraction the binding activities (Fig. 1B, bar graph at the bottom of the gel) of complexes C1 and C2 were reduced to −56−50%, whereas the binding activity of complex C3 was enriched in −100% (compare in Fig. 1B, lanes 2 and 3 with lane 5). When the extracts were tested for the presence of an RNA component that might indicate association of the T. brucei telomerase holoenzyme with the G-rich DNA telomeric oligonucleotide, only complex C3 was 100% sensitive to RNase A pre-treatment (Fig. 1B, lane 6). In pilot experiments, varying amounts (0.2–5 μg) of S100 extracts were tested with 28 fmol of 5’ end-labeled T2AG3 probes, at room temperature or on ice in the presence or absence of RNase A or oligonucleotide competitors (data not shown).

The results in Fig. 1C (gel lanes 1–10 and bar graph) show that complexes C1, C2, and C3 were formed when S100 was mixed with each one of the five 3’ permuted telomeric oligonucleotide (Fig. 1C, lanes 1, 3, 5, 7, and 9). Although not yet understood, complexes C1, C2, and C3 were respectively and preferentially formed when the assays were performed with tel 3 oligonucleotide, tel 1 oligonucleotide, and tel 5 oligonucleotide. The pre-treatment of the extracts with RNase A (Fig. 1C, lanes 2, 4, 6, 8, and 10) showed >95% reduction in binding activity of complex C3 and 10–40% enhancement of complex C2 (bar graph in Fig. 1C). Thus, despite being RNase A-sensitive, complex C3 was stable and could be detected when the binding reaction was done at 0 °C followed by EMSA at 4 °C (Fig. 1, B–D and data not shown).

To characterize the C3 binding activity, EMSAs were done using the DEAE-agarose fraction containing telomerase activity (500 mM) and the 3’ end permuted G-rich telomeric oligonucleotides, as described above for the S100 extracts. Complexes similar to those (C1–C3) shown in Fig. 1C were generated by association of the telomerase fraction (500 mM) with the five permuted oligonucleotides (Fig. 1D, lanes 2, 4, 6, 8, and 10). Pre-treatment of the telomerase fraction with RNase A (Fig. 1D, lanes 3, 5, 7, 9, and 11) also showed that complex C3 formation was highly reduced (−2–4% of binding activity, compared with 65–68% in extracts that were not treated with RNase A), and complex C2 was enhanced in 5–65%, suggesting as in Fig. 1C that complexes C2 and C3 are probably part of a larger complex. In addition, complexes C1, C2, and C3 were not preferentially formed with any of the five 3’ end permuted telomeric oligonucleotides (compare graphs in Fig. 1, C and D) when the telomerase fraction was used as the protein source. Although the formation of complex C3 was reduced ≥50% when tel 3 oligonucleotide was used as probe (Fig. 1D, lane 6 and bar graph). This probably reflects differences in protein composition in S100 extract and the telomerase fraction (see Fig. 4).

The dependence of complex formation on the telomeric oligonucleotide sequence was assessed by competition with increasing concentrations of unlabeled G-strand telomeric sequence and non-telomeric oligonucleotides (the quantitative analysis of the experiments is plotted in Fig. 2A, bottom). S100 extracts were pre-incubated with a mix of labeled oligonucleotide probe and unlabeled competitors (tel 4 and T7, respectively). The
**FIG. 1.** Protein-DNA complex formation by *T. brucei* extracts (S100 and telomerase fraction) incubated with telomeric DNA oligonucleotides permuted at the 3' end. **A**, two-tube TRAP assay (42) to test for the presence of telomerase activity in S100 (lane 1) and 300–500 mM sodium acetate–DEAE fractions (lanes 2–5). Telomerase products were fractionated in a 10% sequencing gel, and enzyme activity was detected by the periodicity of the banding pattern in the 400 and 500 mM DEAE fractions (lanes 3–5) and by the sensitivity to RNase A (lane 5). **B**, EMSA was done with the S100 extract (lane 2) and the 300–500 mM DEAE fractions (lanes 3–6) using 5' end-labeled oligonucleotide tel 4. The assay in lane 1 contained no extract, and in lane 6 the DEAE 500 mM fraction was pre-treated with RNase A. The complexes formed were designated C1, C2, and C3. **C**, EMSA using S100 and the end-labeled oligonucleotides tel 1 (lanes 1 and 2), tel 2 (lanes 3 and 4), tel 3 (lanes 5 and 6), tel 4 (lanes 7 and 8), and tel 5 (lanes 9–11). The extracts were pre-treated with RNase A in lanes 2, 4, 6, 8, and 10. Lane 11 shows an assay done without extract. **D**, telomerase-positive fraction (DEAE 500 mM) incubated with the telomeric oligonucleotides tel 1 (lanes 1–3), tel 2 (lanes 4 and 5), tel 3 (lanes 6 and 7), tel 4 (lanes 8 and 9), and tel 5 (lanes 10 and 11). The EMSA conditions were the same as in Fig. 1B. Lanes 3, 5, 7, 9, and 11 show assays in which extracts were pre-treated with RNase A. In B, C, and D the amount of protein-forming complexes is expressed as the percentage of binding activity as described under “Experimental Procedures.”
concentrations of competitor used were 10 (0.28 pmol) and 50 (1.4 pmol) times greater than the amount of labeled probe (28 fmol of labeled tel 4). As shown in Fig. 2A (lanes 4 and 5), the formation of the three labeled complexes was completely prevented by high concentrations (1.4 pmol) of unlabeled tel 4. The binding activity of complex C1 was reduced in 98% by, respectively, 0.28 and 1.4 pmol of oligonucleotide T7 (Fig. 2A, lanes 6 and 7), suggesting that C1 is probably more specific for the T7 sequence. High concentrations of T7 oligonucleotide (1.4 pmol) reduced in almost 90% the formation of labeled complex C2, indicating that similar binding affinities are observed with both tel 4 and T7 oligonucleotides (Fig. 2A, compare lanes 2 and 7), implying the lack of sequence specificity. In contrast, the percentage of binding activity of complex C3 remained unal-

Fig. 2. C3 is specific for the G-rich telomeric strand of T. brucei. The amount of shifted complexes in A and B is expressed as the percentage of binding activity as described under “Experimental Procedures.” A, EMSA using S100 and tel 4 as a probe, under the same conditions as in Fig. 1, and with competitors (lanes 4–7). Unlabeled tel 4 (lanes 4 and 5) and T7 (lanes 6 and 7) were used at concentrations of 0.28 pmol (10 times) and 1.4 pmol (50 times). The reaction mixtures were run on 6% non-denaturing gels in 0.5× TBE at 4°C. No competitor (NC) was added in lane 1. Complex C3 was completely eliminated when the extracts were pre-treated with RNase A (lane 1). Two unlabeled T. cruzi subtelomeric oligonucleotides CL9RC (lanes 3 and 4) and CL9F2 (lanes 5 and 6) were used as nonspecific competitors in the same amounts as in A. No competitor (0×) was added in lane 1. In A and B, the binding activity in the presence of unlabeled competitors showed p < 0.0001 compared with assays performed in the absence of competitors (Chi-Square and Fisher’s Exact Test). C, binding of C3 is specific for the G-rich telomeric strand of T. brucei. EMSAs were done with the telomerase fraction and 5′-end-labeled oligonucleotides tel 1 (lanes 1 and 2), tel C (lanes 3 and 4), Tet (lane 5 and 6), and T. brucei double-strand (ds) telomeric DNA (lane 7). RNase A pre-treatment eliminated complex C3 (lane 2), but other protein complexes formed with tel C (lane 4) and Tet (lane 6). The amounts of telomerase-positive fraction, labeled oligonucleotides, and the reaction conditions are described under “Experimental Procedures.”
Table I

| Single-stranded oligonucleotides used in EMSA and UV cross-linking |
|---------------------------------------------------------------|
| tel 1 5'–GTAGGGTATTAGGTTAGG–3' |
| tel 2 5'–TAGGTTAGGTTAGGTTAGG–3' |
| tel 3 5'–GGTGATTAGGTTAGGTTAGG–3' |
| tel 4 5'–TTAGGGTATTAGGTTAGG–3' |
| tel 5 5'–GTAGGGTATTAGGTTAGG–3' |
| tel C 5'–CCCTAAACTCCACTAAACTC–3' |
| OvhF 5'–CTGGCGCTGGTATTAGGTTAGGTTAGG–3' |
| OvhR 5'–GTAATGCGGACCCG–3' |
| T7 5'–GTAATACGACTCACTATAGG–3' |
| CLSP2 5'–TGATTCGCGCTGCTGGT–3' |
| CLSRC 5'–TCATCCCTCGCAGTTATAC–3' |
| tel IRNA 5'–GGUGGUGGGUGGGUGGG–3' |
| Tet 5'–GTTGGGGTGGGGTTAGG–3' |

*OvhF is a chimera oligonucleotide of M13 forward and a 17-mer sequence of the 5'-TTAGGG,TTAGGG-3' repeat.
*OvhR is an M13 reverse oligonucleotide.

G-overhang Telomeric Proteins in T. brucei

A 50 to 100 molar excess of unlabeled tel 1 or tel 5 oligonucleotides was used in cross-competition assays with the 500 mM sodium acetate fraction (Fig. 2B and data not shown). All three labeled complexes (C1, C2, and C3) were efficiently inhibited by both oligonucleotides (Fig. 2B, lane 2 and data not shown). Two nonspecific unlabeled oligonucleotides (CL9RC and CL9F2; see Table I) based on sequences from a T. cruzi subtelomeric region (38) were used in these assays to ensure that the DNA binding was specific for T. brucei telomeric DNA. Both nonspecific oligonucleotides (CL9RC and CL9F2) efficiently inhibited complex C1, reduced the binding activity of complex C2 in 60–89% (Fig. 2B, lanes 3–6) and enhanced in 8.5–30% the binding activity of complex C3 (Fig. 3B, lanes 3–6). Binding assays were also done with a C-stranded telomeric oligonucleotide, a duplex DNA formed in vitro by tel 3 and tel C, and an oligonucleotide (Tet) based on the T. thermophila telomeric sequence (Fig. 2C, lanes 3–7). The only complex formed with all of the above single-stranded substrates migrated with the mobility of C1 (Fig. 2C, lanes 1–6). Complexes C2 and C3 were formed only with tel 1 oligonucleotide (Fig. 2C, lane 1) and were confirmed by the sensitivity of C3 to RNase A (Fig. 2C, lane 2). The RNase A-sensitive complex formed with tel C (Fig. 2C, lanes 3 and 4) had a very different mobility from C3 and most likely also had a different composition from that of the C3 complex formed with tel 1 (data not shown). One of the complexes formed with the T. thermophila sequence also appeared to be RNase A-sensitive although it migrated differently from complex C3 (Fig. 2C, compare lanes 5 and 6 with lane 2). There was no complex formation with fully double-stranded T. brucei telomeric DNA (Fig. 2C, lane 7).

Complex C3 Binds Specifically to a Partially Duplex 3’ G-overhang Consisting of 17 Nucleotides of the G-rich T. brucei Telomeric Strand—The interaction of some telomere end-binding proteins is specific for a free 3’ terminus or for a G-rich 3’ single-strand telomere extension from duplex DNA (3’ G-overhangs; see Refs. 9–11). We constructed a DNA fragment containing a 3’ G-overhang that was incubated with the 500 mM sodium acetate telomerase fraction (see “Experimental Procedures” for DNA constructs). Fig. 3A shows that three complexes were formed in the presence of this G-overhang construct (lanes 4–6). Relative to the single-stranded oligonucleotide binding profile, the corresponding C1 complex was under-represented in the G-overhang DNA profile (Fig. 3A, lanes 4 and 5). In contrast, a corresponding complex C3 was formed when the G-overhang DNA construct was used as a probe (Fig. 3A, compare lanes 2 and 4). When the 500 mM sodium acetate fraction was pre-treated with RNase A and subsequently incubated with the G-overhang DNA, the band corresponding to C3 in the single-strand DNA complex was not formed with the partial duplex DNA (see complex C3* in Fig. 3A, lane 5). This suggested that similar protein-DNA complexes were formed when the telomerase fraction was incubated with the partially duplex G-overhang DNA and the single-stranded tel 1 oligonucleotide. Despite their apparent similarity to the single-stranded complexes, the complexes formed with the partial duplex DNA (G-overhang), and the telomerase fraction showed a different mobility in 6% native gels probably because of the different DNA conformation acquired by G-overhang DNA when compared with single-stranded DNA.

Complex C3 Is Highly Sensitive to Chemical Nuclease Treatment—According to Harrington et al. (46) the complex formed by T. thermophila telomerase and a G-strand telomeric DNA oligonucleotide is completely eliminated by pre-treating the extracts with RNase. Thus, to further confirm the presence of an RNA component in complex C3, the telomerase fraction (DEAE 500 mM sodium acetate fraction) was subjected to hydroxyl radical treatment. Hydroxyl radicals can be generated in vitro by the action of hydrogen peroxide on (FeII)(EDTA)2– and can attack either single- or double-stranded DNA in a sequential manner. The hydroxyl radical can also alter protein-DNA and protein-RNA interactions by changing the nucleic acid conformation or other physical interactions that might affect the sensitivity of possible substrates to nuclease treatment (47). Before the binding reactions were done, the hydroxyl radicals generated in the mixture containing the telomerase fraction were quenched by adding glycerol. In control experiments, complex C3 was found to be highly sensitive to up 0.03% hydrogen peroxide (the binding activity was reduced in 90.3–96.1%), regardless of whether the binding reaction using the telomerase fraction occurred with tel 1 DNA or tel 1 RNA (Fig. 3B, compare lanes 4 and 9 with lanes 5 and 10). Because hydroxyl radicals were generated at 0.003% hydrogen peroxide, this was the concentration chosen for subsequent experiments. Only complex C3 was almost 100% destroyed by hydroxyl radical treatment, as seen with tel 1 DNA (Fig. 3B, lane 6 and bar graph) and tel 1 RNA (Fig. 3B, lane 11 and bar graph) probes. The sensitivity of complex C3 to RNase A (Fig. 3B, lane 3) and hydroxyl radicals (Fig. 3B, lanes 6 and 11) is consistent with the presence of an RNA component in the complex.

The Sizing and Complexity of C3-associated Proteins Was Estimated by UV Cross-linking Assays—UV cross-linking experiments were done to assess the size and complexity of the protein species associated with complex C3. After UV irradiation, proteins cross-linked to tel 1 in the S100 extract (Fig. 4, lanes 1 and 2) and the telomerase (500 mM) fraction (Fig. 4, lanes 3–8) were separated on 4–20% gradient gels. Four prominent protein bands of ~18–50 kDa were obtained for S100 (all molecular masses mentioned include the 18-nucleotide tel 1 oligonucleotide). Differences in the profile of the protein bands between the S100 extract and the 500 mM fraction most likely reflected variations in the purity of the fractions (compare in Fig. 4, lanes 1 and 2 with lanes 5, 6, and 8). In the RNase A pre-treated and UV cross-linked complexes formed with S100 extracts and tel 1 (Fig. 4, lane 2) there was a complexed band of ~18 kDa which, contrary to its counterpart in the telomerase fraction (Fig. 4, lane 6), formed even after pre-treating the S100 extract with RNase A and probably involved a protein found only in the S100 extract.
EMSAs with the telomerase-positive fraction (500 mM) and tel 1 oligo-
trich telomeric terminus and involves an RNA component.

G-overhang DNA. In RNase A-sensitive complex formed with the telomerase fraction and the
lanes 1 and 2, S100 extract; lanes 3–8, telomerase fraction; lane 3 shows a binding reaction that was not exposed to UV light. Extracts were treated with
RNase A prior to binding to tel 1 oligonucleotide (lanes 2 and 6) or
DNase I (lane 7). A 50-molar excess (1.4 pmol) of a nonspecific unlabeled competitor was used in the binding reaction in lane 4. Lane 8 shows a reaction in which bovine serum albumin was not used during the binding assay. The arrows indicate the nucleoprotein complex bands (18 and 25 kDa) that possibly correspond to complex C3.

To further characterize the proteins in the 500 mM sodium
acetate fraction that may participate in complex C3 formation
(Fig. 4, lanes 3–8), extracts were pre-incubated with a 50-molar
excess of unlabeled CL9RC followed by a binding reaction with
5’ end-labeled tel 1 before UV irradiation (Fig. 4, lane 4). Only
the proteins bands corresponding to ~25 and 18 kDa were not
inhibited and cross-linked to tel 1 oligonucleotide. The 25- and
18-kDa bands disappeared when the telomerase fraction was
pre-treated with RNase A (Fig. 4, lane 6), indicating that these
bands are probably part of C3. No protein bands were detected
when tel 1 oligonucleotide was used as an unlabeled competitor
(data not shown). The telomerase fraction was pre-treated with
1 unit of DNase I to eliminate the possibility of contamination
by nicked telomeric DNA (Fig. 4, lane 7). In other EMSA (data
not shown), we observed that only C3 remained when this
protein fraction was treated with DNase I, before or after
oligonucleotide binding or after UV irradiation. This result
shows that DNase I does not interfere with the formation of C3
(data not shown), which was also independent of the absence
or presence of bovine serum albumin in the binding reaction (Fig. 4, lane 8).

Formation of Complex C3 with the RNA Oligonucleotide Cog-
nate of the G-strand Telomere Sequence—G-strand telomere-
binding proteins that bind more avidly to the RNA cognate of
the DNA oligonucleotide have been described in
Chlamydomonas (48), Saccharomyces cerevisiae (10, 11, 21), and T. ther-
mophila (12). In Fig. 5, A and B, the relative binding affinity of
the components of complex C3 associated with tel 1 RNA (Fig.
5A, lanes 1–4) and tel 1 DNA (Fig. 5A, lanes 5–8), using the
telomerase-positive (500 mM sodium acetate) DEAE fraction,
was judged by the amount of labeled complex and by cross-

the telomerase positive fraction underwent hydroxyl radical (‘OH)
treatment prior to binding assays. 0.03% of hydrogen peroxide (HP)
interfered with the binding of the complexes (lanes 4 and 9). Hydroxyl
radical (‘OH) treatment at a lower concentration of peroxide (0.003%) is
shown in lanes 6 (tel 1 DNA) and 11 (tel 1 RNA). Lanes 2 and 8 show the
normal binding reaction with tel 1 DNA and tel 1 RNA, respectively.
Lane 3 shows extract pre-treated with RNase A, and lanes 5 and 10
show pre-treatment with 0.003% hydrogen peroxide followed by binding
to tel 1 DNA and tel 1 RNA, respectively. Free tel 1 DNA and free tel 1
RNA are shown in lanes 1 and 7, respectively. No reagents (NR) were
added to the binding reaction.

![Diagram](http://www.jbc.org/)
G-overhang Telomeric Proteins in T. brucei

In the presence of unlabeled competitors, a preparative 12% gel and separated by two-dimensional gel electrophoresis. The gels were silver-stained, and spots corresponding to the above protein bands were compared with their labeled counterparts after exposure to x-ray film (see Fig. 6A, on the left, gel 1 and gel 2 and on the right corresponding autoradiographs). Because of the low abundance of proteins in the spots, in both cases, only some spots were subject to in-gel digestion with endoproteinase Lys-C and subsequent characterization by MALDI-TOF MS. Fig. 6B shows diagrams of the corresponding peptide maps of spots 2, 4, 5, and 8 from the 25-kDa band and spot 2 from the 18-kDa band. The peptide peaks are indicated in Daltons, and the underlined peaks were utilized in searches of trypanosome and protein data bases using the Protein Prospector MS-FIT analysis program (Baker, P. R. and Clauser, K. R.; www.prospector.ucsf.edu). No homologues were found for these peptides in the data bases searched.

**DISCUSSION**

In many organisms, telomeric G-rich single-stranded DNA provides sites for telomere-binding proteins that protect the chromosome end terminus or recruit telomerase action (23, 28, 49). Telomere maintenance in most eukaryotes requires several telomere/telomerase-associated proteins, in addition to the reverse transcriptase protein (telomerase reverse transcriptase) and the telomerase RNA (TER) (23). Proteins with overlapping features and functions have been found in yeast, ciliates, and humans (12, 50–55), indicating that telomere structure is organized and maintained by generally conserved

**Fig. 5.** Complex C3 binds an RNA version of the G-rich telomere sequence. A, EMSAs were done as in Fig. 1 using the telomerase-positive fraction and the cognate tel 1 RNA (lanes 1–4) or tel 1 DNA oligonucleotides (lanes 5–8). Cross-competition assays were performed using 50 (1.4 pmol) or 100-fold excess (2.8 pmol) of unlabeled tel 1 DNA oligonucleotide and labeled tel 1 RNA (lanes 3 and 4) or unlabeled tel 1 RNA and labeled tel 1 DNA as the probe (lanes 7 and 8). In lanes 1 and 5, no extract was added to the binding reactions. Arrows are pointed to the right for complexes C1, C2, and C3. The amount of shifted complexes was quantified for each experiment and expressed as the percentage of binding activity as described under "Experimental Procedures." In the presence of unlabeled competitors p < 0.01 compared with assays performed in the absence of competitors (Fisher's Exact Test). ss, single-strand. NC, no competitors added. B, upper panel, EMSAs were done after exposing to UV light the binding reactions using the telomerase fraction and tel 1 RNA; lower panel, the corresponding UV cross-linked protein/RNA complexes were separated by SDS-PAGE in a 10% gel. In lanes 3 and 11, RNase A was added before binding; in lanes 4 and 12, the RNase A inhibitor (RNasin) was added at the same time as RNase A before binding, and in lanes 5 and 13, RNase A was added to the reaction after binding but before UV cross-linking. Note that complex C3 was partially protected by RNasin pretreatment. The extracts were incubated with protease K (PK) before binding to tel 1 DNA in lanes 6 and 14 or to tel 1 RNA oligonucleotides in lanes 7 and 15.
evolutionary mechanisms. The yeast EST (ever short telomere) gene products Est1p, Est2, and Est3 (53) are associated with yeast telomerase RNA and telomerase (20) and are involved in the regulation of telomere length. In contrast to Rap1p and Trf1p, double-stranded telomere-binding proteins that negatively regulate telomerase action (13, 16, 18), the G-strand telomere-binding proteins Cdc13p and Est1p (10, 11, 21), are considered positive regulators of telomerase action. Cdc13p protects the end of the chromosomes by functioning as a cap and plays a role in telomere replication by interacting with

FIG. 6. MALDI-TOF MS spectra of complex C3-associated 18- and 25-kDa proteins. A, silver-stained two-dimensional gels (left) and corresponding autoradiographs (right) of the 18- and 25-kDa C3-associated proteins. The numbers assigned correspond to the protein spots subjected to endoproteinase digestion and MALDI-TOF analysis. B, mass spectra of the Lys-C-digested spot 2 (i), spot 4 (ii), spot 5 (iii), and spot 8 (iv) of the 25-kDa protein and spot 2 (v) of the 18-kDa protein. In all profiles, the peaks inside boxes correspond to the masses (Daltons) used in the data base searches with Protein Prospector MS-FIT. The peaks corresponding to keratin are marked with filled circles (on the right). Peptide standards (Std.) of 1297.43 and 2867.97 Da were used to calibrate the mass scale.
telomerase through binding to Est1p (11, 50). Est1p is associated with TER and requires a free, single-stranded 3’ terminus, as well as the presence of Cdc13p, to promote the recruitment of telomerase to telomeres (10, 20, 50).

The three protein-DNA complexes described here were formed in vitro using trypanosome G-rich single-strand telomeric repeat sequences permuted at the 3’ end and fractions of a T. brucei extract that contained telomerase activity (42). Complex C1 was the most nonspecific, because it (i) was eluted throughout the DEAE-agarose column fractionation profile, (ii) appeared as the same complex formed by binding the C-strand telomere sequence and the T. thermophila G-strand telomeric DNA, and (iii) was competed by all non-telomeric sequences tested. Protein(s) of complex C2 also eluted in all DEAE-agarose fractions (300–500 mM) but was(ere) less concentrated in the telomerase-positive 500 mM fraction. Complex C2 was formed with all tel oligonucleotides and with the ciliate tel 1 RNA version of the G-rich telomere strand, although it was almost totally competed by some non-telomeric oligonucleotides. The possibility that all three complexes (C1, C2, and C3) are the same complex or parts of the same complex that are intimately associated cannot be excluded, because competition with CL9RC and CL9F2 reduced the formation of complexes C1 and C2 and enhanced complex C3. In addition, pre-treating the extracts with RNase A abolished C3 formation and depending on the probe or extract used, enhanced complexes C1 and C2 bands in EMSAs.

Complex C3 showed the most specific binding to the T. brucei G-rich telomere and was the only complex that was not inhibited by any of the non-telomeric oligonucleotides tested. Cross-competition assays showed that complex C3 bound slightly better to an RNA oligonucleotide containing the telomere ciliate tel 1 RNA sequence (tel 1 RNA) than to the corresponding DNA oligonucleotide (tel 1 DNA). As with yeast Est1p, C3 also required a free single-strand 3’ region for binding, indicating that it shared properties with Est1p and other G-rich single-stranded telomere end-binding proteins of yeast (10, 50–53), ciliates (12, 22, 54), and humans (51, 55). The T. brucei telomerase is able to elongate tel 1 RNA, which is consistent with the possibility that, like p80/p95 of T. brucei ciliates (12, 22, 54), and humans (51, 55). The region of T. brucei EST1 is intimately associated with TER and requires a free, single-stranded 3’ RNA similar to that of Cdc13p and Est1p and plays an important role in telomere maintenance (50, 56). In the more extensively characterized telomerase of Tetrahymena, the ability of the ribonucleoprotein enzyme to bind telomeric oligonucleotides was distinct from primer elongation efficiency (46). This resemblance is also consistent with the possibility that, as proposed for Est1p (10, 50) and p80/p95 (12), C3 provides a specific site for primer binding on the telomerase holoenzyme that is distinct from the telomerase active site (23).

A preliminary attempt to identify the 18- and 25-kDa protein components of complex C3 by MALDI-TOF MS analysis, although not totally negative, revealed some of the inconveniences of dealing with low abundance proteins, including contamination of the preparation with keratin. The large scale preparation of C3 should overcome these technical problems and allow protein sequencing by mass spectrometry for the identification of these proteins. The lack of homologous proteins based on the data base searches suggests that the C3-associated proteins are novel T. brucei proteins.

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A *Trypanosoma brucei* Protein Complex That Binds G-overhangs and Co-purifies with Telomerase Activity

Maria Isabel N. Cano, Julie Johnson Blake, Elizabeth H. Blackburn and Nina Agabian

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