Published online September 30, 2004

5214–5222 *Nucleic Acids Research, 2004, Vol. 32, No. 17*
doi:10.1093/nar/gkh864

Biochemical properties of *Trypanosoma cruzi* telomerase

Denise P. Muñoz and Kathleen Collins*

Department of Molecular and Cell Biology, 16 Barker Hall, University of California at Berkeley, Berkeley, CA 94720-3204, USA

Received August 11, 2004; Revised and Accepted September 15, 2004

ABSTRACT

Trypanosomatid parasite infections have a devastating impact on human health. Little is known about the requirements for parasite growth during any stage of their complex, multi-host life cycle. In most eukaryotic organisms, sustained cell proliferation requires telomerase-dependent telomere length maintenance. Here we investigate the regulation and biochemical features of telomerase from *Trypanosoma cruzi*, the causative agent of Chagas disease. We found that *T.cruzi* telomerase is active in extracts from multiple developmental stages of the parasite life cycle. Detailed characterization of the enzymatic properties of telomerase using epimatigote-stage extract revealed a unique combination of substrate specificities, consistent with the evolutionary divergence of trypanosomes from previously established model systems for telomerase biochemical characterization. Results from partial purification of *T.cruzi* telomerase suggest that the catalytically active enzyme is a large ribonucleoprotein complex and that the internal RNA template has an atypical, cytosine-rich permutation. These results expand our understanding of telomerase enzymology and should encourage the development of parasite-specific telomerase inhibitors as a method for disease therapy.

INTRODUCTION

All cells that proliferate indefinitely must replicate a complete genome content. Because the ends of linear chromosomes are not fully duplicated by DNA-dependent DNA polymerases, organisms with linear chromosomes require an additional, end-specific replication mechanism. Without adequate end-replication, the progressive loss of terminal sequences will compromise genomic integrity (1,2). In different cell types, short telomeres can trigger an irreversible exit from the cell cycle (replicative senescence), cell death from genomic instability (crisis) or programmed cell death (apoptosis). The lack of telomere maintenance in normal human somatic cells typically results in replicative senescence (3) whereas inhibition of telomere maintenance in human cancer cells leads to cell death (4–6). In single-celled organisms such as ciliates and yeasts, inadequate telomere length maintenance dramatically inhibits cell proliferation (7,8).

Different chromosome end-specific replication mechanisms have evolved to maintain cellular and viral genomes (9). In eukaryotes, end replication by telomerase may be the most phylogenetically widespread mechanism of telomere maintenance. Telomerase activity has been identified in organisms including ciliates (10), vertebrates (11), yeasts (12), plants (13), nematodes (14) and insects (15). Gene mutations or knockouts that deplete or eliminate telomerase activity compromise telomere length maintenance in single-celled (16,17) and multicellular organisms as well (18–21). The resulting telomere erosion inhibits proliferative renewal and long-term organismal viability. In yeast and cultured mammalian cells, these phenotypes of telomerase deficiency can be suppressed by activation of a telomerase-independent alternative telomere maintenance (ALT) mechanism (22). The ALT pathway can efficiently immortalize cultured mouse fibroblasts (23), but mice themselves cannot remain healthy and reproduce without telomerase enzyme for more than a few generations (19).

The telomerase ribonucleoprotein (RNP) holoenzyme complex elongates chromosome 3′ ends by addition of a species-specific, simple-sequence DNA repeat, using defined residues within its integral RNA component as template (24,25). The gene encoding the telomerase RNA component has been cloned from numerous eukaryotes and one virus (26,27). The length and primary sequence of these RNAs vary greatly. Within ciliates, vertebrates and some yeasts, phylogenetic comparisons have revealed some elements of group-specific conserved secondary structure (28–32). The functions of most of the conserved RNA motifs remain to be determined, but they can influence enzyme properties or recruit telomere binding or other regulatory proteins (26,33). Active telomerase RNPs also incorporate a conserved protein subunit, telomerase reverse transcriptase (TERT) (34), which contains the active site motifs shared by viral reverse transcriptase (RT) enzymes (35).

Most studies of telomerase enzymology have been done in ciliate, mammalian or yeast model systems. All these organisms represent recent branches of the eukaryotic phylogenetic

*To whom correspondence should be addressed. Tel: +1 510 643 1598; Fax: +1 510 643 6334; Email: kcollins@socrates.berkeley.edu
Correspondence may also be addressed to Denise P. Muñoz. Email: DPMunoz@lbl.gov
Present address:
Denise P. Muñoz, Lawrence Berkeley National Lab, 1 Cyclotron Road Mail Stop 74R0157, Berkeley, CA 94720, USA

tree in comparison with kinetoplastid species of parasitic protozoa (36). The latter group includes vertebrate pathogens that cause severe, deleterious impacts on world health and economy. In humans, *Trypanosoma brucei* causes sleeping sickness and *T.cruzi* causes Chagas disease (37). The chromosomal telomeric repeat of *T.brucei* (38,39) and *T.cruzi* (40) is the same as that of vertebrates, with repeats of 5′TTAGGG3′ toward a 3′ terminus. In addition, *T.brucei* chromosome ends form terminal t-loop structures similar to those that cap mammalian chromosomes (41,42). Cell extracts from replicative stages of the *T.brucei* parasite life cycle have active telomerase, supporting an important role for telomerase-dependent telomere length maintenance *in vivo* (43).

There are several questions uniquely relevant to a consideration of telomerase-dependent telomere maintenance in trypanosomatids. These parasites have life cycles with distinct replicative and infective forms that are specific for a particular insect or animal host. Parasite telomere length maintenance could therefore be restricted to a particular developmental stage. Also, despite the evolutionary divergence between parasite and host, their telomerase enzymes synthesize the same telomeric repeat sequence. To better understand telomerase enzymes from human parasites, we have characterized *T.cruzi* telomerase activity. We found that telomerase activity was detectable in multiple stages of the *T.cruzi* life cycle. Characterization of the epimastigote-stage telomerase activity revealed a unique combination of nucleotide, primer and template specificities, distinct from those of the human telomerase enzyme. Partial purification results imply that active *T.cruzi* telomerase is a large holoenyzme complex with an atypical permutation of its RNA template. These studies extend the phylogenetic range of telomerase characterization and should encourage future efforts toward development of telomerase inhibitors for clinical use in controlling the severity and spread of trypanosomatid parasite infection.

MATERIALS AND METHODS

Parasite strains and culture

Epimastigotes were *T.cruzi* Tulahuen strain of Tul 2 stock. They were grown as described previously (44) and were harvested during log-phase at $\sim 8 \times 10^7$ cells/ml. The four major developmental stages of the CL Brener clone were purified to homogeneity as assessed by microscopy. They were kindly provided by Dr J. J. Cazzulo (Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín, San Martín, Provincia de Buenos Aires, Argentina).

Parasite extracts

Parasites were collected by centrifugation, washed once in ice-cold phosphate-buffered saline, pelleted at 8000 *g* for 10 min at 4°C and resuspended at 10^8 parasites/30 µl of ice-cold lysis buffer (10 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 20 mM *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E-64), 5 mM ß-mercaptoethanol, 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), 10% glycerol). The suspension was incubated at 4°C for 30 min and then centrifuged at 40 000 *g* for 90 min. The supernatant was collected and stored in aliquots at −80°C. Protein concentration was measured by the Bradford method and typically ranged from 1 to 2 mg/ml.

Telomerase activity

Up to 10 µg of total protein was incubated for 2 h at 28–30°C in telomerase assay reaction buffer (50 mM Tris–acetate pH 8.0, 10 mM spermidine, 5 mM ß-mercaptoethanol, 2 mM $MgCl_2$). Unless indicated otherwise, assays contained 800 µM dATP and TTP with 2–8 µM unlabeled dGTP and 1.5 µM [α-^{32}P]dGTP (800 Ci/mmol). Some extract samples were pretreated for 10 min at 37°C with RNase A. Activity assay reactions were stopped by addition of TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA), extracted with phenol:chloroform: isoamyl alcohol, precipitated with ammonium acetate and ethanol and then resolved by denaturing gel electrophoresis. AZT-TP was purchased from Moravek Biochemicals. For markers of oligonucleotide migration, oligonucleotides were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP for 30 min at 37°C.

Telomerase activity across the life cycle of *T.cruzi* was assayed as described above with 5′-biotinylated telomeric repeat primer as substrate [b-$(G_3T_2A)_3$]. After incubation, 10 µl of streptavidin agarose beads (Sigma) were mixed with each sample and allowed to bind for 10 min. The assay reaction was then stopped as described above and the beads were collected by centrifugation, washed twice in TE, resuspended in Proteinase K mix (10 mM Tris–HCl pH 7.5, 0.5% SDS, 1.5 µg Proteinase K per reaction) and incubated for 30 min at 37°C. Products were extracted, precipitated and resolved as described above.

Gel filtration

Two hundred microliters of epimastigote-stage extract was loaded onto a Superose 6 column (Pharmacia), equilibrated and run in T2MG (20 mM Tris–HCl pH 8.0, 1 mM $MgCl_2$, 10% glycerol) with 50 mM KCl. The sample was collected in 40 µl fractions except for the void volume, which was collected in one fraction of 400 µl. Telomerase activity was assayed as described above, using 20 µl of each fraction in a final 30 µl reaction volume. Molecular weight standards (Bio-Rad) were thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa) and equine myoglobin (17 kDa).

Oligonucleotide-based affinity purification

Streptavidin agarose beads (15 µl per binding reaction) were blocked as described previously (45) and charged with 7 µg of biotinylated oligonucleotide as indicated. Two hundred microliter of extract adjusted to 0.4 M KCl was mixed with 15 µg of each of two competitor DNA oligonucleotides (TCCGCCTTTTTC and TCCGCCTTTTTCGGGCACGGGAACG, in which the underlined region is 2′-*O*-methyl RNA), incubated at 4°C for 15 min and centrifuged 15 min at 10 000 *g* at 4°C. The supernatant was collected and mixed with charged beads; binding was then carried out at room temperature for 90 min. Subsequently, the beads were washed five times in T2MG with 0.4 M KCl and once with T2MG without salt. Finally, the beads were resuspended in T2MG with 10 µg of the elution oligonucleotide and incubated at room temperature for 1 h.

RESULTS

Telomerase activity in different stages of the *T.cruzi* life cycle

Previous studies have shown that telomerase activity could be detected in extracts from cells of one stage of the *T.brucei* and *Leishmania* life cycles (43) and replicative stages of *Plasmodium falciparum* (46,47). Activity could be readily detected using the two-step TRAP assay, in which telomerase-dependent primer elongation products were amplified by PCR before detection (48). Telomerase could also be detected in *T.brucei* extract using a direct primer extension assay, if extract was first partially purified by fractionation on DEAE agarose. The life cycle of *T.cruzi* includes four developmental stages (49). Two stages of the life cycle are infective but non-replicative: the bloodstream trypomastigote (in the blood of the vertebrate host) and the metacyclic trypomastigote (in the rectum of the insect vector). Two additional stages of the life cycle are non-infective but replicative: the epimastigote (in the gut of the insect vector) and the amastigote (inside the vertebrate cell). We tested telomerase activity in extracts from cells representing these four developmental forms of *T.cruzi* using a direct primer extension assay.

Detection of *T.cruzi* telomerase activity in whole cell extracts other than the epimastigote stage was obscured by a high background of extract nucleic acid radiolabeling (data not shown). We therefore used a 5′-biotinylated telomeric repeat oligonucleotide as primer. After incubation of the extract in the presence of dATP, TTP and ^{32}P-dGTP, the primer and any radiolabeled extension products were recovered by binding to streptavidin beads. In this way, we could reliably detect RNase-sensitive primer extension products in whole cell extracts from at least three of the four stages of the *T.cruzi* life cycle (Figure 1). We used numerous criteria to verify if these radiolabeled DNAs were the products of a telomerase enzyme activity, including the demonstration of dependence of product synthesis on the combination of dNTPs required to complete a telomeric repeat (see below). It is important to note that the quantitative comparison of telomerase activity across different stages is imprecise, because each extract could contain different non-specific enzyme inhibitors.

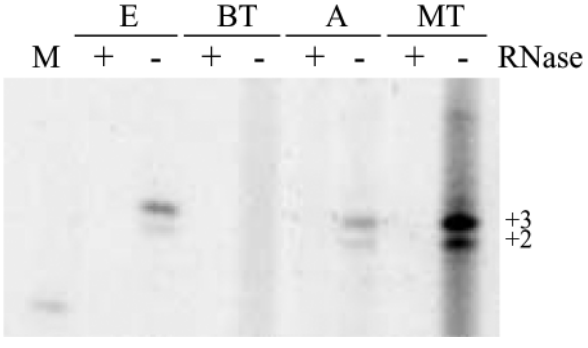


Figure 1. Telomerase activity in cell extracts from different stages of the *T.cruzi* life cycle. Life cycle stages are: E, epimastigote; BT, bloodstream trypomastigote; A, amastigote; and MT, metacyclic trypomastigote. Telomerase activity was measured by direct primer extension with TTP, dATP and ^{32}P-dGTP using 5 μM of the 5′ biotinylated DNA oligonucleotide b-$(G_3T_2A)_3$. Lane M contains a 5′ phosphorylated telomeric repeat primer without biotin as a migration marker.

Low repeat addition processivity at all dNTP concentrations

We used the epimastigote-stage extract to characterize the biochemical properties of *T.cruzi* telomerase in greater detail. Telomerase products from this extract could be visualized without post-assay fractionation, due to the low level of non-specific nucleic acid radiolabeling performed by the extract. Importantly, we found no difference in epimastigote-stage telomerase activity features when comparing whole cell extract and partially purified enzyme (some data below; additional data not shown).

To test the nucleotide requirements for *T.cruzi* telomerase activity, we assayed elongation of the single-stranded DNA primer $(TAG_3T)_3$ in the presence of a fixed amount of radiolabeled dGTP and a titration of unlabeled dATP, TTP or dGTP (Figure 2A). Telomerase-dependent synthesis at the 3′ end of this primer should require addition of TTP and dATP prior to addition of radiolabeled dGTP, due to the permutation of the telomeric repeat. As expected, no product synthesis was detectable in the absence of either dATP (lane 1) or TTP (lane 7). Under our standard assay conditions of 1.5 μM radiolabeled dGTP and 2 μM unlabeled dGTP, addition of 2 μM dATP (in saturating TTP concentration) or 10 μM TTP (in saturating dATP concentration) was sufficient to detect product synthesis (lanes 2–6, 8–12). Telomerase enzymes of other species also show this slightly higher K_m for incorporation of pyrimidine nucleotides (50).

Telomerases from most species can dissociate an elongated product from the 5′ end of the RNA template and reposition it at the template 3′ end for another round of repeat synthesis. Ciliate or vertebrate enzymes can accomplish this repeat addition processivity using either dGTP-independent or dGTP-dependent mechanisms (51–53). For example, the dGTP-dependent repeat addition processivity of recombinant *Tetrahymena* telomerase is stimulated by 5–10 μM dGTP, a much higher concentration than that required for efficient dGTP addition within a repeat (53). The addition of increasing amounts of unlabeled dGTP to *T.cruzi* telomerase activity assays had no stimulatory impact on repeat addition processivity, instead causing only a reduction in dGTP specific activity and therefore product intensity (Figure 2A, lanes 13–18). No dGTP-dependent stimulation of repeat addition processivity was detected with any of the several different *T.cruzi* telomerase preparations (data not shown).

Recombinant *Tetrahymena* telomerase repeat addition processivity can also be stimulated by dGMP or dGDP (53). To test whether dGDP or dGMP stimulated the processivity of *T.cruzi* telomerase, we added increasing concentrations of each of these nucleotides or dGTP into reaction mixtures containing 1.5 μM radiolabeled dGTP and the primer $(G_3T_2A)_3$ (Figure 2B). No stimulation of nucleotide or repeat addition processivity was observed. The presence of dGDP inhibited dGTP incorporation in a manner similar to the effect of dilution with unlabeled dGTP (lanes 6–10, 11–15), whereas dGMP had no effect (lanes 1–5). Elongation products from the first round of repeat addition always remained predominant. We conclude that DNA synthesis by *T.cruzi* telomerase has an inherently low repeat addition processivity *in vitro*. Although the active site appears to bind dGDP in competition with dGTP, dGMP is not similarly effective as a competitor.

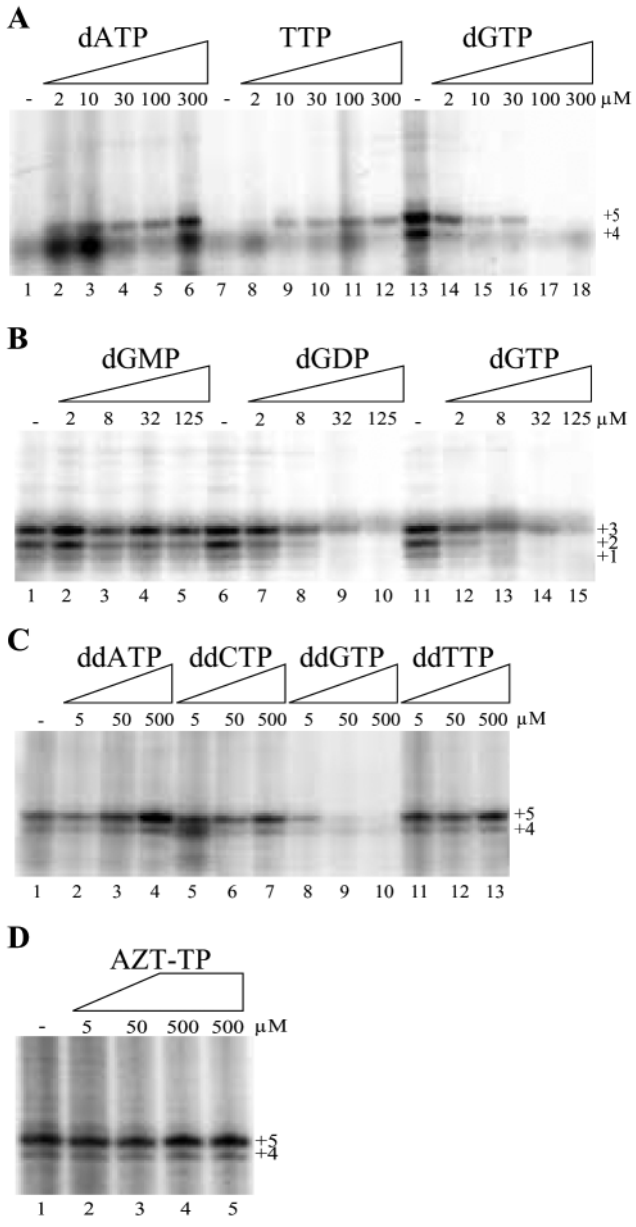


Figure 2. Nucleotide dependence of primer elongation. Product migration is indicated by the number of nucleotides added to the primer 3′ end. (**A**) Requirements for dNTPs. Telomerase activity was assayed with 1 μM of primer $(TAG_3T)_3$ using telomerase partially purified by gel filtration. All reactions contained 1.5 μM ^{32}P-dGTP. In the dATP titration, TTP was fixed at 200 μM and unlabeled dGTP at 5 μM. In the TTP titration, dATP was fixed at 200 μM and unlabeled dGTP at 5 μM. In the dGTP titration, dATP and TTP were present at 200 μM and unlabeled dGTP was added as indicated. (**B**) Effect of deoxyguanosine nucleotides. Telomerase activity was assayed with 1 μM of primer $(G_3T_2A)_3$ using telomerase partially purified by gel filtration. All reactions contained 1.5 μM ^{32}P-dGTP with additional unlabeled dGMP, dGDP or dGTP as indicated. (**C** and **D**) Effect of ddNTPs and AZT-TP. Telomerase activity was assayed with 5 μM of primer $(TAG_3T)_3$ using telomerase in whole cell extract. All reactions contained 5 μM ^{32}P-dGTP, 50 μM dATP, 50 μM TTP and the additional unlabeled nucleotide as indicated.

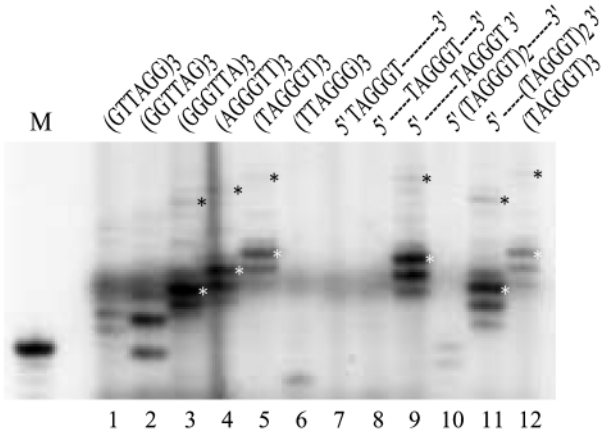
This suggests that phosphate groups provide some of the interaction affinity for nucleotides at the *T.cruzi* telomerase active site.

Telomerases from other species are inhibited *in vitro* and *in vivo* by chain-terminating nucleotide analogs including dideoxynucleoside triphosphates (ddNTPs) and azidothymidine triphosphate (AZT-TP) (46,54,55). Human and *Tetrahymena* telomerases are inhibited by lower concentrations of ddGTP than ddTTP or AZT-TP, likely due to the preferential binding of guanosine in the active site (50). *Tetrahymena* telomerase efficiently incorporates ddNTPs, halting its otherwise highly processive mode of product synthesis. Although ddNTP incorporation by human telomerase can be detected in the absence of the cognate dNTP, activity inhibition under standard assay conditions occurs by ddNTP competition for dNTP binding at the active site (11,55). This difference accounts for the higher ddNTP concentration required to inhibit human telomerase than the *Tetrahymena* enzyme (54,55). To determine if *T.cruzi* telomerase is susceptible to inhibition by nucleotide analogs, we assayed activity in reactions with fixed dNTP concentrations and a titration of one of the four ddNTPs (Figure 2C) or AZT-TP (Figure 2D). Completion of first-repeat synthesis using the primer $(TAG_3T)_3$ requires addition of 1 TTP, 1 dATP and 3 dGTP nucleotides in sequence. Product synthesis remained detectable in reactions with similar concentrations of dGTP and ddGTP (Figure 2C, lane 8) but was clearly inhibited in reactions with ddGTP at 50 or 500 μM concentration, in 10- or 100-fold excess of dGTP (lanes 9 and 10). This result indicates that chain-terminating nucleotide analogs can inhibit *T.cruzi* telomerase.

In contrast, the addition of ddATP, ddCTP, ddTTP (Figure 2C, lanes 2–4, 5–7, 11–13) or AZT-TP (Figure 2D) had little or no inhibitory effect when added at even 500 μM concentration. This represents an up to 10-fold excess over dATP or TTP. These results suggest that *T.cruzi* telomerase does not incorporate AZT-TP in preference to TTP, as observed for human telomerase (55). The inhibition by ddATP or ddTTP could be less for *T.cruzi* telomerase than for human telomerase due to less favorable ddNTP binding in the active site, less product dissociation in the absence of the proper dNTP substrate or other factors.

Product dependence on the sequence of the primer 3′ end

Telomerase enzymes from most organisms can extend DNA primers that deviate from the telomeric repeat sequence. In ciliates, this may reflect the physiological requirement for telomerase activity at sites of new telomere formation as well as at established telomeres (56). To address the DNA interaction specificity of *T.cruzi* telomerase, we first compared the elongation of a set of 18 nt primers representing different permutations of the telomeric repeat. This assesses the ability of different primer 3′ end sequences to pair in correct register with the template and to place different template positions in the active site. Consistent with the copying of a fixed-permutation template to its end, the permuted primers generated predominant products of different lengths (Figure 3, lanes 1–6). The primers with 3′ permutations G_3T_2A, AG_3T_2, TAG_3T were elongated most efficiently, with the addition of up to 3, 4 or 5 nt, respectively (lanes 3–5; white asterisks mark first-repeat synthesis products, black asterisks mark products elongated by addition of a second repeat). This represents synthesis to complete the 3′ permutation T_2AG_3, as judged by comparison with the end-labeled primers (lane M) and changes in the product



putative template

Lanes	3' ——————CCCAAUCCC——————5'
1	5' GTTAGGGTTAGGGTTAGG 3'
2	5' GGTTAGGGTTAGGGTTAG 3'
3	5' GGGTTAGGGTTAGGGTTA 3'
4	5' AGGGTTAGGGTTAGGGTT 3'
5, 12	5' TAGGGTTAGGGTTAGGGT 3'
6	5' TTAGGGTTAGGGTTAGGG 3'
7	5' TAGGGT---------- 3'
8	5' ------TAGGGT------ 3'
9	5' ----------TAGGGT 3'
10	5' TAGGGTTAGGGT------ 3'
11	5' ------TAGGGTTAGGGT 3'

Figure 3. Primer sequence requirements. The elongation of telomeric and partially telomeric sequence primers was assayed using telomerase partially purified by gel filtration with 1 μM of an 18 nt primer. Six-nucleotide blocks of non-telomeric sequence within the chimeric primers contained AATCCG (5′ end or 5′ of two consecutive blocks) and TCGAGC (3′ end or 3′ of two consecutive blocks). White asterisks indicate complete first-repeat addition products; black asterisks indicate complete second-repeat addition products. The products of one chimeric primer migrated offset from other products of the same length due to sequence context (lane 11). M lane contains 5′ phosphorylated telomeric repeat primer $(TAG_3T)_3$ as a migration marker. A summary of inferred primer alignment with the putative *T.cruzi* telomerase RNA template is also shown.

profile upon omission of individual dNTPs (Figure 2A; additional data not shown).

The products of primers with other telomeric repeat permutations were weak in intensity (GT_2AG_2; Figure 3, lane 1), aberrant (G_2T_2AG; lane 2) or almost undetectable (T_2AG_3; lane 6). The same pattern of product DNAs was obtained using independent primer preparations and different primer concentrations (data not shown). These results reveal that potential substrates with different 3′ ends have different elongation efficiencies, at least for *in vitro* activity. Overall, these findings also suggest a template permutation for *T.cruzi* telomerase RNA of 5′CCCUAACCC3′, such that dNTP addition to the template 5′ end completes the synthesis of products ending in T_2AG_3-3′. This predicted template permutation parallels that predicted for *T.brucei* telomerase using similar criteria (43) and differs from the vertebrate telomerase RNA template permutation 5′CUAACCCU(AA)3′ (29). The atypically cytosine-rich sequence of the trypanosome telomerase RNA templates would be expected to increase the stability of the template-product hybrid formed upon synthesis to the template 5′ end. Like yeast telomerase RNA templates that are atypically long, the cytosine-rich trypanosome telomerase RNA templates may serve to strengthen the association of telomerase and its telomere substrates.

We next examined the elongation of chimeric primers, composed of mixed telomeric and non-telomeric sequence. One repeat of the efficiently elongated permutation TAG_3T was placed at a 5′, internal or 3′ location within a primer, 18 nt in total length. Elongation was not detected for the 5′ or internal telomeric repeat primers (Figure 3, lanes 7 and 8). An 18 nt primer with two telomeric repeats at its 5′ end was also not elongated, except possibly following cleavage of the input primer to remove the non-telomeric 3′ end (lane 10, note the products of less than input primer length; see below). In contrast, elongation of a primer with a single 3′ repeat (Figure 3, lane 9) was as or more efficient than elongation of a 3′-repeat primer of the same permutation (lanes 5 and 12). A primer with two 3′ repeats was also efficiently elongated (lane 11; note that the complete first and second repeat addition products had altered mobility due to the unique non-telomeric sequence at the primer 5′ end). These results indicate that for *T.cruzi* telomerase, a 5′ telomeric-sequence cassette does not substitute for a telomeric-sequence 3′ end in directing efficient elongation.

We also assayed the elongation of primers with an entirely non-telomeric sequence. Consistent with the elongation of chimeric primers lacking a telomeric-sequence 3′ end, relatively little product was detectable in assays of an entirely non-telomeric sequence primer (Figure 4A, lanes 1–5). Very limited product synthesis was observed, even in reactions with 10 μM of primer (lane 5), whereas telomeric repeat primer elongation was detected with primer concentrations as low as 10 nM (lane 7). To examine whether inefficient elongation derives at least in part from a relatively weak binding of non-telomeric sequence DNA to the *T.cruzi* telomerase enzyme, we added 10 μM of two different non-telomeric sequence primers or other competitors to reactions containing 1 μM of the efficiently elongated telomeric primer $(TAG_3T)_3$. Although the inefficiently elongated telomeric repeat primer $(T_2AG_3)_3$ could inhibit $(TAG_3T)_3$ elongation completely (Figure 4B, compare lanes 1 and 2), neither of the non-telomeric sequences did so (compare lanes 1, 3 and 4).

From all of the primer specificity studies described above, we conclude that *T.cruzi* telomerase has little if any reliance on protein-dependent DNA anchoring interactions in establishing the sequence specificity of substrate elongation *in vitro*. Instead, substrate specificity is determined predominantly or entirely by base-pairing between the primer 3′ end and the RNA template. An atypical, cytosine-rich permutation of the *T.cruzi* telomerase RNA template could mediate a particularly high-affinity substrate binding by hybridization, reducing the importance of an independent, protein-based anchor site interaction.

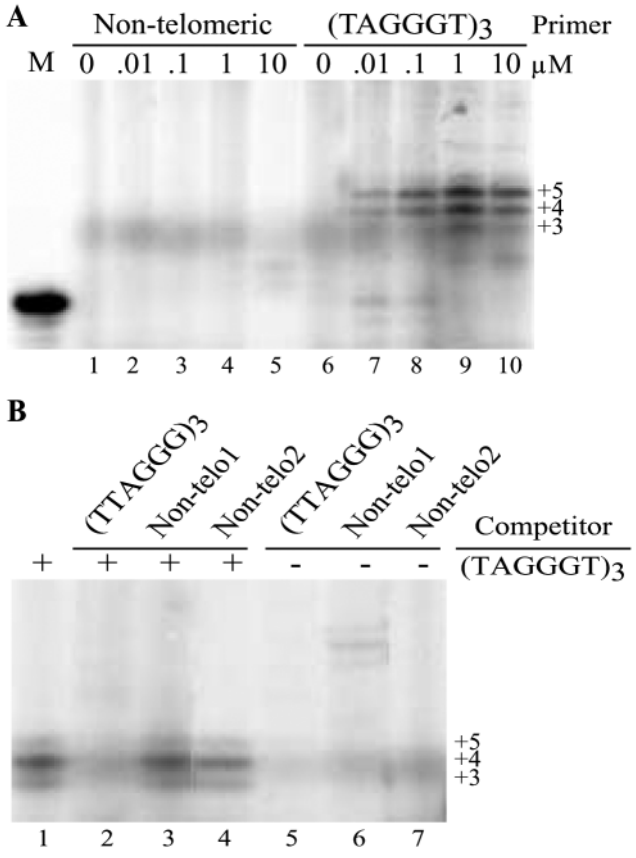


Figure 4. No non-telomeric sequence primer elongation or interference. Product migration is indicated by the number of nucleotides added to the primer 3′ end. (**A**) Direct elongation. Activity was assayed using telomerase partially purified by gel filtration with the indicated concentrations of non-telomeric (AATCCGTCGAGCAGAGTT) or telomeric $(TAG_3T)_3$ sequence primers. M lane contains 5′ phosphorylated telomeric repeat primer $(TAG_3T)_3$ as a migration marker. (**B**) Elongation competition. Activity was assayed using telomerase partially purified by gel filtration in the presence (lanes 1–4) or absence (lanes 5–7) of 1 μM of $(TAG_3T)_3$ with 10 μM of each indicated competitor. Competitor oligonucleotides are $(T_2AG_3)_3$ in lanes 2 and 5, Non-telo1 (AGCCACTATCGACTACGCGGGG) in lanes 3 and 6, and Non-telo2 (AATCCGTCGAGCAGAGTT) in lanes 4 and 7. Some elongation of Non-telo1 is detectable in lane 6, likely due to the primer –GGGG-3′ end.

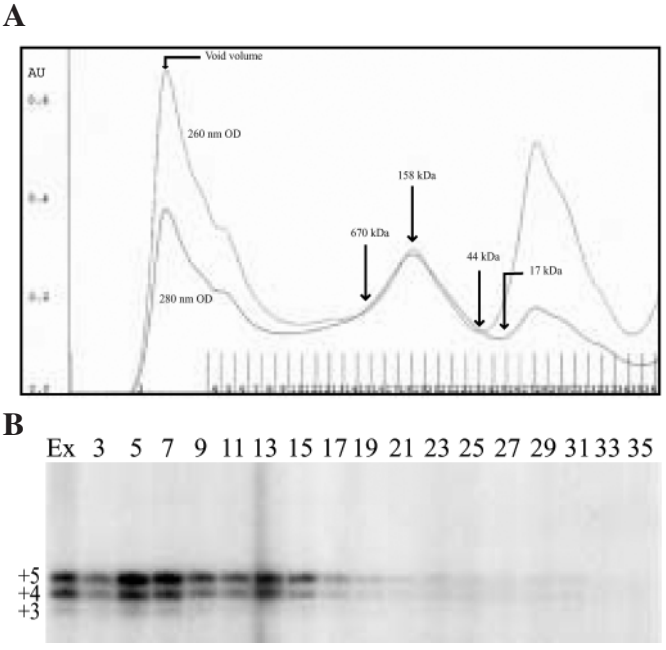


Figure 5. Estimation of *T.cruzi* telomerase RNP mass. (**A**) Chromatogram from extract fractionation on a Superose 6 gel filtration column. Absorbance (AU) is shown at 280 and 260 nm. Arrows indicate the peak fractions for recovery of protein standards with the given molecular weights. (**B**) Activity assay using 5 μl of the load extract (Ex) or 20 μl of the indicated column fractions. Assays contained 1 μM of $(TAG_3T)_3$. Product migration is indicated by the number of nucleotides added to the primer 3′ end.

Nucleolytic cleavage activity

In addition to primer elongation, telomerases can also catalyze primer or product cleavage (12,57–60). Cleavage is most frequently evident in the appearance of radiolabeled products equal to or less than input primer length. Cleavage appears to be catalyzed by the same active site as DNA synthesis and is therefore stimulated by conditions that inhibit dNTP addition, including low dNTP concentration, primer-template mismatch and primer positioning at the template 5′ end. We detected a potential primer cleavage activity of *T.cruzi* telomerase in reactions with specific telomeric repeat permutations, with chimeric primers bearing 5′ telomeric repeats and a non-telomeric 3′ end and with low primer concentrations (Figure 3, lanes 2, 6 and 10; Figure 4A, lanes 7 and 8). These findings are consistent with a telomerase-associated nuclease activity that can remove 3′ residues from template-hybridized substrates.

A large *T.cruzi* telomerase complex

To gain insight on the physical composition of the active *T.cruzi* telomerase RNP, we examined its fractionation by gel filtration (see Materials and Methods). We compared the profiles of bulk protein and nucleic acid detected by absorbance ratio at 280 and 260 nm (Figure 5A) with telomerase activity assayed by primer extension (Figure 5B). Telomerase activity was recovered predominantly in fractions near the void volume of the column, representing molecular weights well above the 670 kDa standard. This finding suggests that the *T.cruzi* telomerase enzyme can be a large RNP complex, as found for endogenous telomerase RNPs in budding and fission yeasts and in various vertebrate cells (35,61–63). We also investigated *T.cruzi* telomerase RNP mass using glycerol gradient sedimentation (data not shown). Enzyme dilution in the gradient was sufficient to prevent reliable detection of activity by direct primer extension assay of gradient fractions. After concentration of pools of gradient fractions by binding to and elution from DEAE agarose, telomerase activity was detected only in the pool from higher glycerol concentrations than the pool that contained the 670 kDa standard.

Template-directed oligonucleotide affinity purification

Ciliate and mammalian telomerase RNPs have been partially purified by exploiting the accessibility of the RNA template to oligonucleotide hybridization (45,63). Although this affinity purification strategy may perturb some of the architecture of the holoenzyme complex, it can allow the recovery of an active enzyme. The primer elongation and competition studies

described above suggested a putative 1.5-repeat *T.cruzi* telomerase RNA template 5′CCCUAACCC3′, directing the synthesis of products with a T_2AG_3 3′ end. However, preferential product dissociation does not necessarily occur at the template 5′ end (12,51,64). To test the putative *T.cruzi* telomerase RNA template permutation using an independent method, we compared affinity purification using four different sequences of 2′-*O*-methyl RNA bound via biotin to streptavidin beads. We compared the binding of *T.cruzi* telomerase to a 2′-*O*-methyl RNA complementary to its putative template sequence (an oligonucleotide ending in $G_3U_2AG_3$-3′), a 2′-*O*-methyl RNA complementary to human telomerase RNA template (an oligonucleotide ending in $GU_2AG_3U_2AG$-3′) and to two additional oligonucleotides directed against the *Tetrahymena* telomerase RNA template as controls (Figure 6). Supernatants from affinity purification with control 2′-*O*-methyl RNA oligonucleotides complementary to the *Tetrahymena* telomerase RNA template retained levels of activity consistent with the input extract after dilution and incubation (lanes 6 and 8). In contrast, when *T.cruzi* template-complementary 2′-*O*-methyl RNA oligonucleotides were used, supernatants were depleted for telomerase activity (lanes 2 and 4). Complete depletion was attained with the RNA complementary to the *T.cruzi* template permutation predicted from activity assays described above.

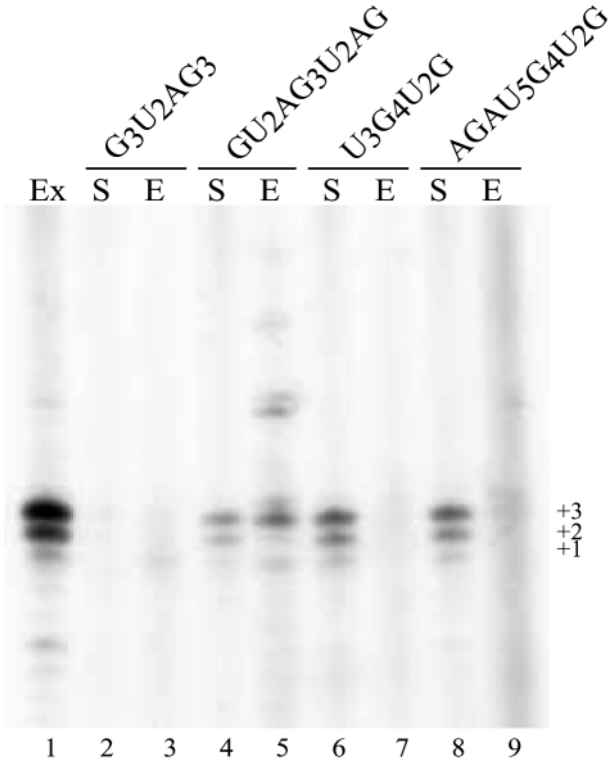


Figure 6. Oligonucleotide-based affinity purification. Affinity purification was accomplished using 5′ biotinylated oligonucleotides with chimeric DNA and 2′-*O*-methyl RNA sequences (underlined sequences represent 2′-*O*-methyl RNA): lanes 2 and 3, b-$TC_2GC_2T_5$CGGGUUAGGG (complementary to the putative *T.cruzi* template permutation); lanes 4 and 5, b-$TC_2GC_2T_5$CGUUAGGGUUAG (complementary to the human telomerase RNA template permutation); lanes 6 and 7, b-$TC_2GC_2T_5$CUUUGGGGUUG (complementary to the *Tetrahymena* telomerase RNA template region); and lanes 8 and 9, b-$TC_2G_2T_5$CAGAUUUUUGGGGUUG (complementary to the *Tetrahymena* telomerase RNA template region and 3′ flanking sequence). Elution was performed using DNA oligonucleotides complementary to the entire length of the biotinylated oligonucleotides including both 2′-*O*-methyl RNA and DNA. Activity was assayed with 5 μM of $(G_3T_2A)_3$ using extract (lane 1, Ex) or supernatants (even lanes, S) and elutions (odd lanes, E) from affinity purification with the indicated 2′-*O*-methyl RNA sequences. The extract sample was not diluted into the high ionic strength binding buffer of the supernatant samples (see Materials and Methods). Product migration is indicated by the number of nucleotides added to the primer 3′ end.

We tested the competitive elution of *T.cruzi* telomerase from the 2′-*O*-methyl RNA affinity resins using DNA oligonucleotides complementary to the entire length of the 2′-*O*-methyl RNA sequence and the DNA linker between 2′*O*-methyl RNA and 5′ biotin (see Figure 6 legend). The elution oligonucleotides were not elongated and did not inhibit telomerase activity, consistent with data from the assays of other primers with non-telomeric sequence 3′ ends described above (data not shown). No activity was eluted from the resins with 2′-*O*-methyl RNA oligonucleotides complementary to the *Tetrahymena* telomerase RNA template (Figure 6, lanes 7 and 9). Telomerase activity was eluted from the 2′-*O*-methyl RNA resin complementary to the human telomerase RNA template (lane 5). The repeat addition processivity of this sample was the maximum observed among all *T.cruzi* enzyme preparations, possibly linked to the partial disruption of endogenous telomerase RNP structure observed with this method of purification (44,62). Very little activity was eluted from resin with the predicted *T.cruzi* template-complementary oligonucleotide (lane 3) despite depletion of activity from the supernatant (lane 2). One interpretation of these results is that the interaction of *T.cruzi* telomerase with the 2′-*O*-methyl RNA sequence $G_3U_2AG_3$ occurs with such high affinity that competitive elution cannot occur under the gentle conditions used here to retain enzyme activity. A simple loss of activity seems less likely, given that activity was recovered in other samples that were analyzed in parallel. We suggest that these results provide physical evidence in support of the *T.cruzi* telomerase RNA template permutation 5′CCCUAACCC3′ hypothesized from primer elongation and competition studies.

DISCUSSION

Trypanosoma cruzi is the etiological agent of Chagas disease, also known as American trypanosomiasis. Understanding the biochemical requirements for parasite growth in different stages of the life cycle should provide avenues for the discovery of much needed, more effective drugs. Because human telomerase inhibition can halt cancer cell proliferation (65,66), *T.cruzi* telomerase inhibition could likewise halt parasite proliferation. Our detection of telomerase activity in extracts from cells of multiple life cycle stages, including cells not directly capable of proliferation, is surprising. Perhaps the need for rapid proliferation in some stages of the life cycle obliges the presence of constitutively active enzyme. The ability to detect activity by direct primer extension suggests that these parasites produce relatively high levels of telomerase, despite the presence of <100 chromosomes per cell (67,68).

The characterization of *T.cruzi* telomerase activity revealed a combination of features distinct from the ciliate, mammalian and yeast enzymes studied to date. Unlike telomerases from most organisms, *T.cruzi* telomerase appears to recognize DNA substrates dependent only on the presence of a telomeric repeat

at the 3′ end. This finding *in vitro* is consistent with the observed requirement *in vivo* for telomere-like sequences at sites of new telomere formation on linear DNA molecules in *T.brucei* (69). We also find that primer 3′ ends with different telomeric repeat permutations have different elongation efficiencies. Primers ending with T_2AG_3-3′ were not well elongated and were preferentially subject to nucleolytic cleavage. A primer with this 3′ permutation inhibited the elongation of other telomeric repeat primers, as if it has annealed with the template so effectively as to prevent template copying. Even *Euplotes aediculatus* telomerase, which like *T.cruzi* telomerase has a permutation-dependent affinity for telomeric repeat primers (70), demonstrates less bias against elongation of the 3′ permutation predicted to hybridize at the template 5′ end (51).

Multiple repeat additions by *T.cruzi* telomerase can be detected in some activity assays, using either whole cell extract or partially purified enzyme. A vast excess of unreacted primer is present in all of the activity assay conditions used here, so the multiple-repeat addition products must derive from a limited extent of repeat addition processivity. During *T.brucei* replication in a vertebrate host, telomeres undergo a net elongation of ~10 bp per population doubling (71). The addition of only one or a few telomeric repeats per telomerase-telomere interaction event could be compensated by a large amount of active telomerase enzyme per cell.

Trypanosoma cruzi telomerase activity fractionates with overlap of the void volume of a Superose 6 column, suggesting an RNP complex of substantially >670 kDa. Using mild buffer conditions for gel filtration and glycerol gradient sedimentation, telomerase RNP complexes have been observed to fractionate at 250–500 kDa for active ciliate enzymes from *Tetrahymena thermophila* and *E.aediculatus* (45,72,73); 280, 550, 1600 kDa and larger for catalytically distinct *Euplotes crassus* RNPs (74) and >700 kDa for endogenous yeast and mammalian telomerase complexes (35,61–63). The large mass of the active *T.cruzi* telomerase RNP is likely to derive in part from the incorporation of holoenzyme proteins required for RNA stability or regulation at the telomere, as characterized in other organisms (24,75). The large RNP mass could also arise in part from TERT and/or telomerase RNA multimerization (34).

Affinity purification with template-complementary 2′-*O*-methyl RNA oligonucleotides achieved a substantial purification of *T.cruzi* telomerase. Optimal depletion of telomerase activity from extracts was obtained using the RNA $G_3U_2AG_3$-3′ rather than the longer RNA $GU_2AG_3U_2AG$-3′. This observation provides the strongest evidence to date for a putative RNA template, modeled with 1.5 repeat total length, of 5′CCCUAACCC3′. Our efforts to purify and identify *T.cruzi* telomerase RNA or TERT have been unsuccessful thus far, but we hope that future efforts toward these goals will be facilitated by the biochemical studies described here.

ACKNOWLEDGEMENTS

We thank Dr J. J. Cazzulo for parasite extracts and members of the Collins laboratory for discussion and comments on the manuscript. This study was supported by a fellowship from Universidad de Buenos Aires (D.P.M.) and a New Investigator in Pharmacological Sciences grant from the Burroughs Wellcome Fund (K.C.).

REFERENCES

1. Harrington,L. (2004) Those dam-aged telomeres. *Curr. Opin. Genet. Dev.*, **14**, 22–28.
2. Chan,S.R. and Blackburn,E.H. (2004) Telomeres and telomerase. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.*, **359**, 109–121.
3. Bodnar,A.G., Ouellette,M., Frolkis,M., Holt,S.E., Chiu,C., Morin,G.B., Harley,C.B., Shay,J.W., Lichtsteiner,S. and Wright,W.E. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science*, **279**, 349–352.
4. Hahn,W.C., Stewart,S.A., Brooks,M.W., York,S.G., Eaton,E., Kurachi,A., Beijersbergen,R.L., Knoll,J.H.M., Meyerson,M. and Weinberg,R.A. (1999) Inhibition of telomerase limits the growth of human cancer cells. *Nature Med.*, **5**, 1164–1170.
5. Zhang,X., Mar,V., Zhou,W., Harrington,L. and Robinson,M.O. (1999) Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev.*, **13**, 2388–2399.
6. Herbert,B.-S., Pitts,A.E., Baker,S.I., Hamilton,S.E., Wright,W.E., Shay,J.W. and Corey,D.R. (1999) Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc. Natl Acad. Sci. USA*, **96**, 14276–14281.
7. Lundblad,V. and Szostak,J.W. (1989) A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell*, **57**, 633–643.
8. Yu,G., Bradley,J.D., Attardi,L.D. and Blackburn,E.H. (1990) *In vivo* alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. *Nature*, **344**, 126–132.
9. de Lange,T. (2004) T-loops and the origin of telomeres. *Nature Rev. Mol. Cell Biol.*, **5**, 323–329.
10. Greider,C.W. and Blackburn,E.H. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell*, **43**, 405–413.
11. Morin,G.B. (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell*, **59**, 521–529.
12. Cohn,M. and Blackburn,E.H. (1995) Telomerase in yeast. *Science*, **269**, 396–400.
13. McKnight,T.D., Fitzgerald,M.S. and Shippen,D.E. (1997) Plant telomeres and telomerases. A review. *Biochemistry* (*Moscow*), **62**, 1224–1231.
14. Magnenat,L., Tobler,H. and Muller,F. (1999) Developmentally regulated telomerase activity is correlated with chromosomal healing during chromatin diminution in *Ascaris suum*. *Mol. Cell. Biol.*, **19**, 3457–3465.
15. Sasaki,T. and Fujiwara,H. (2000) Detection and distribution patterns of telomerase activity in insects. *Eur. J. Biochem.*, **267**, 3025–3031.
16. Singer,M.S. and Gottschling,D.E. (1994) TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science*, **266**, 404–409.
17. Miller,M.C. and Collins,K. (2000) The *Tetrahymena* p80/p95 complex is required for proper telomere length maintenance and micronuclear genome stability. *Mol. Cell*, **6**, 827–837.
18. Blasco,M.A., Lee,H., Hande,M.P., Samper,E., Lansdorp,P.M., DePinho,R.A. and Greider,C.W. (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell*, **91**, 25–34.
19. Lee,H.-W., Blasco,M.A., Gottlieb,G.J., Horner,J.W.I., Greider,C.W. and DePinho,R.A. (1998) Essential role of mouse telomerase in highly proliferative organs. *Nature*, **392**, 569–574.
20. Mitchell,J.R., Wood,E. and Collins,K. (1999) A telomerase component is defective in the human disease dyskeratosis congenita. *Nature*, **402**, 551–555.
21. Riha,K., McKnight,T., Griffing,L. and Shippen,D. (2001) Living with genome instability: plant responses to telomere dysfunction. *Science*, **291**, 1797–1800.
22. Colgin,L.M. and Reddel,R.R. (1999) Telomere maintenance mechanisms and cellular immortalization. *Curr. Opin. Genet. Dev.*, **9**, 97–103.
23. Hande,M.P., Samper,E., Lansdorp,P. and Blasco,M.A. (1999) Telomere length dynamics and chromosome instability in cells derived from telomerase null mice. *J. Cell Biol.*, **144**, 589–601.
24. Harrington,L. (2003) Biochemical aspects of telomerase function. *Cancer Lett.*, **194**, 139–154.

25. Greider,C.W. (1995) Telomerase biochemistry and regulation. In Blackburn,E.H. and Greider,C.W. (eds.), *Telomeres*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 35–68.
26. Chen,J.L. and Greider,C.W. (2004) Telomerase RNA structure and function: implications for dyskeratosis congenita. *Trends Biochem. Sci.*, **29**, 183–192.
27. Fragnet,L., Blasco,M.A., Klapper,W. and Rasschaert,D. (2003) The RNA subunit of telomerase is encoded by Marek's disease virus. *J. Virol.*, **77**, 5985–5996.
28. Romero,D.P. and Blackburn,E.H. (1991) A conserved secondary structure for telomerase RNA. *Cell*, **67**, 343–353.
29. Chen,J.-L., Blasco,M.A. and Greider,C.W. (2000) Secondary structure of vertebrate telomerase RNA. *Cell*, **100**, 503–514.
30. Tzfati,Y., Knight,Z., Roy,J. and Blackburn,E.H. (2003) A novel pseudoknot element is essential for the action of a yeast telomerase. *Genes Dev.*, **17**, 1779–1788.
31. Dandjinou,A.T., Levesque,N., Larose,S., Lucier,J.F., Abou Elela,S. and Wellinger,R.J. (2004) A phylogenetically based secondary structure for the yeast telomerase RNA. *Curr. Biol.*, **14**, 1148–1158.
32. Zappulla,D.C. and Cech,T.R. (2004) Yeast telomerase RNA: a flexible scaffold for protein subunits. *Proc. Natl Acad. Sci. USA*, **101**, 10024–10029.
33. Blackburn,E.H. (2000) The end of the (DNA) line. *Nat. Struct. Biol.*, **7**, 847–850.
34. Kelleher,C., Teixeira,M.T., Forstemann,K. and Lingner,J. (2002) Telomerase: biochemical considerations for enzyme and substrate. *Trends Biochem. Sci.*, **27**, 572–579.
35. Lingner,J., Hughes,T.R., Shevchenko,A., Mann,M., Lundblad,V. and Cech,T.R. (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science*, **276**, 561–567.
36. Sogin,M.L. (1991) Early evolution and the origin of eukaryotes. *Curr. Opin. Genet. Dev.*, **1**, 457–463.
37. Barrett,M.P., Burchmore,R.J., Stich,A., Lazzari,J.O., Frasch,A.C., Cazzulo,J.J. and Krishna,S. (2003) The trypanosomiases. *Lancet*, **362**, 1469–1480.
38. Van der Ploeg,L.H.T., Liu,A.Y.C. and Borst,P. (1984) Structure of the growing telomeres of trypanosomes. *Cell*, **36**, 459–468.
39. Blackburn,E.H. and Challoner,P.B. (1984) Identification of a telomeric DNA sequence in *Trypanosoma brucei*. *Cell*, **36**, 447–457.
40. Freitas-Junior,L.H., Porto,R.M., Pirrit,L.A., Schenkman,S. and Scherf,A. (1999) Identification of the telomere in *Trypanosoma cruzi* reveals highly heterogeneous telomere lengths in different parasite strains. *Nucleic Acids Res.*, **27**, 2451–2456.
41. Muñoz-Jordan,J.L., Cross,G.A.M., de Lange,T. and Griffith,J.D. (2001) t-loops at trypanosome telomeres. *EMBO J.*, **20**, 579–588.
42. Griffith,J.D., Comeau,L., Rosenfield,S., Stansel,R.M., Bianchi,A., Moss,H. and de Lange,T. (1999) Mammalian telomeres end in a large duplex loop. *Cell*, **97**, 503–514.
43. Cano,M.I.N., Dungan,J.M., Agabian,N. and Blackburn,E.H. (1999) Telomerase in kinetoplastid parasitic protozoa. *Proc. Natl Acad. Sci. USA*, **96**, 3616–3621.
44. Bontempi,E., Franke de Cazzulo,B., Ruiz,A. and Cazzulo,J.J. (1984) Purification and properties of an acidic protease from epimastigotes of *Trypanosoma cruzi*. *Comp. Biochem. Physiol.*, **77**, 599–604.
45. Lingner,J. and Cech,T.R. (1996) Purification of telomerase from *Euplotes aediculatus*: requirement of a primer 3′ overhang. *Proc. Natl Acad. Sci. USA*, **93**, 10712–10717.
46. Bottius,E., Bakhsis,N. and Scherf,A. (1998) *Plasmodium falciparum* telomerase: *de novo* telomere addition to telomeric and nontelomeric sequences and role in chromosome healing. *Mol. Cell. Biol.*, **18**, 919–925.
47. Aldous,W.K., Martin,R.K. and Kyle,D.E. (1998) Stage specific detection and inhibition studies of *Plasmodium falciparum* telomerase. *Mol. Biochem. Parasitol.*, **95**, 281–285.
48. Kim,N.W., Piatyszek,M.A., Prowse,K.R., Harley,C.B., West,M.D., Ho,P.L.C., Coviello,G.M., Wright,W.E., Weinrich,S.L. and Shay,J.W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**, 2011–2015.
49. Marsden,P.D. (1971) South American trypanosomiasis (Chagas' disease). *Int. Rev. Trop. Med.*, **4**, 97–121.
50. Collins,K. (1999) Ciliate telomerase biochemistry. *Annu. Rev. Biochem.*, **68**, 187–218.
51. Hammond,P.W. and Cech,T.R. (1997) dGTP-dependent processivity and possible template switching of *Euplotes* telomerase. *Nucleic Acids Res.*, **25**, 3698–3704.
52. Maine,I.P., Chen,S. and Windle,B. (1999) Effect of dGTP concentration on human and CHO telomerase. *Biochemistry*, **38**, 15325–15332.
53. Hardy,C.D., Schultz,C.S. and Collins,K. (2001) Requirements for the dGTP-dependent repeat addition processivity of recombinant *Tetrahymena* telomerase. *J. Biol. Chem.*, **276**, 4863–4871.
54. Strahl,C. and Blackburn,E.H. (1994) The effects of nucleoside analogs on telomerase and telomeres in *Tetrahymena*. *Nucleic Acids Res.*, **22**, 893–900.
55. Strahl,C. and Blackburn,E.H. (1996) Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. *Mol. Cell. Biol.*, **16**, 53–65.
56. Melek,M. and Shippen,D.E. (1996) Chromosome healing: spontaneous and programmed *de novo* telomere formation by telomerase. *Bioessays*, **18**, 301–308.
57. Collins,K. and Greider,C.W. (1993) Nucleolytic cleavage and non-processive elongation catalyzed by *Tetrahymena* telomerase. *Genes Dev.*, **7**, 1364–1376.
58. Melek,M., Greene,E.C. and Shippen,D.E. (1996) Processing of non-telomeric 3′ ends by telomerase: default template alignment and endonucleolytic cleavage. *Mol. Cell. Biol.*, **16**, 3437–3445.
59. Oulton,R. and Harrington,L. (2004) A human telomerase-associated nuclease. *Mol. Biol. Cell*, **15**, 3244–3256.
60. Huard,S. and Autexier,C. (2004) Human telomerase catalyzes nucleolytic primer cleavage. *Nucleic Acids Res.*, **32**, 2171–2180.
61. Lue,N.F. and Peng,Y. (1997) Identification and characterization of a telomerase activity from *Schizosaccaromyces pombe*. *Nucleic Acids Res.*, **25**, 4331–4337.
62. Nakayama,J., Saito,M., Nakamura,H., Matsuura,A. and Ishikawa,F. (1997) TLP1: a gene encoding a protein component of mammalian telomerase is a novel member of WD repeats family. *Cell*, **88**, 875–884.
63. Schnapp,G., Rodi,H.-P., Rettig,W.J., Schnapp,A. and Damm,K. (1998) One-step affinity purification protocol for human telomerase. *Nucleic Acids Res.*, **26**, 3311–3313.
64. Ye,A.J. and Romero,D.P. (2002) A unique pause pattern during telomere addition by the error-prone telomerase from the ciliate *Paramecium tetraurelia*. *Gene*, **294**, 205–213.
65. Shay,J.W. and Wright,W.E. (2002) Telomerase: a target for cancer therapeutics. *Cancer Cell*, **2**, 257–265.
66. Corey,D.R. (2002) Telomerase inhibition, oligonucleotides, and clinical trials. *Oncogene*, **21**, 631–637.
67. Henriksson,J., Porcel,B., Rydaker,M., Ruiz,A., Sabaj,V., Galanti,N., Cazzulo,J.J., Frasch,A.C. and Pettersson,U. (1995) Chromosome specific markers reveal conserved linkage groups in spite of extensive chromosomal size variation in *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.*, **73**, 63–74.
68. Cano,M.I., Gruber,A., Vazquez,M., Cortes,A., Levin,M.J., Gonzalez,A., Degrave,W., Rondinelli,E., Zingales,B., Ramirez,J.L. *et al.* (1995) Molecular karyotype of clone CL Brener chosen for the *Trypanosoma cruzi* genome project. *Mol. Biochem. Parasitol.*, **71**, 273–278.
69. Horn,D., Spence,C. and Ingram,A.K. (2000) Telomere maintenance and length regulation in *Trypanosoma brucei*. *EMBO J.*, **19**, 2332–2339.
70. Hammond,P.W. and Cech,T.R. (1998) *Euplotes* telomerase: evidence for limited base-pairing duing primer elongation and dGTP as an effector of translocation. *Biochemistry*, **37**, 5162–5172.
71. Bernards,A., Michels,P.A.M., Lincke,C.R. and Borst,P. (1983) Growth of chromosome ends in multiplying trypanosomes. *Nature*, **303**, 592–597.
72. Wang,H. and Blackburn,E.H. (1997) *De novo* telomere addition by *Tetrahymena* telomerase *in vitro*. *EMBO J.*, **16**, 866–879.
73. Collins,K., Kobayashi,R. and Greider,C.W. (1995) Purification of *Tetrahymena* telomerase and cloning of genes encoding the two protein components of the enzyme. *Cell*, **81**, 677–686.
74. Greene,E.C. and Shippen,D.E. (1998) Developmentally programmed assembly of higher order telomerase complexes with distinct biochemical and structural properties. *Genes Dev.*, **12**, 2921–2931.
75. Vega,L.R., Mateyak,M.K. and Zakian,V.A. (2003) Getting to the end: telomerase access in yeast and humans. *Nature Rev. Mol. Cell Biol.*, **4**, 948–959.