Purification of telomerase from *Euplotes aediculatus*: Requirement of a primer 3' overhang

JOACHIM LINGNER and THOMAS R. CECH*

Howard Hughes Medical Institute, Department of Chemistry and Biochemistry, University of Colorado, Campus Box 215, Boulder, CO 80309-0215

Contributed by Thomas R. Cech, July 25, 1996

**ABSTRACT**  Telomerase is a ribonucleoprotein enzyme that uses its internal RNA moiety as a template for synthesis of telomeric repeats at chromosome ends. Here we report the purification of telomerase from *Euplotes aediculatus* by affinity chromatography with antisense 2'-O-methyl oligonucleotides, a method that was developed for small nuclear ribonucleoprotein particles (snRNPs). Elution of bound ribonucleoprotein from the antisense oligonucleotide under nondenaturing conditions was achieved by a novel approach, using a displacement oligonucleotide. Polypeptides of 120 kDa and 43 kDa (a doublet) copurify with the active telomerase and appear stoichiometric with telomerase RNA. A simple model for DNA end replication predicts that after semiconservative DNA replication, telomerase will extend the newly synthesized, blunt-ended leading strand. We show that purified *Euplotes* telomerase has no activity with blunt-ended primers. Instead, efficient extension requires 4 to 6 single-stranded nucleotides at the 3' end. Therefore, this model predicts the existence of other activities such as helicases or nucleases that generate a single-stranded 3' end from a blunt end, thus activating the end for telomerase extension.

Telomeres are the DNA–protein complexes that form the ends of eukaryotic chromosomes. Telomeric sequences usually consist of simple repeats rich in G residues in the strand that runs 5' to 3' toward the chromosomal ends (for recent review see ref. 1). The complete replication of the ends of eukaryotic chromosomes poses special problems to the conventional DNA replication machinery. Conventional DNA polymerases cannot prime DNA synthesis *de novo*, but instead require RNA primers which are removed later in replication. Removal of the RNA primer from the lagging-strand end would leave behind a 5'-terminal gap, thus resulting in the loss of sequence, if the parental telomere were blunt ended (2, 3). However, the telomeres whose structures have been analyzed so far are not blunt but have 3' overhangs (4–6). For such molecules, removal of the lagging-strand 5'-terminal RNA primer could regenerate the 3' overhang without loss of information on this side of the molecule; however, loss of sequence information on the leading-strand end would occur, because of the lack of a complementary strand to act as template in the synthesis of a 3' overhang (7, 8).

Although conventional polymerases cannot accurately reproduce chromosomal DNA ends, specialized factors exist to ensure their complete replication. A key component in this process is telomerase. Telomerase is a ribonucleoprotein particle (RNP) and polymerase that uses a portion of its internal RNA moiety as a template for telomere repeat DNA synthesis (9–13; for review see refs. 14 and 15). This enzyme was discovered more than 10 years ago in the single-celled ciliate *Tetrahymena thermophila* (16). Since then, the activity has been detected in numerous eukaryotic organisms. However, ciliated protozoa provide a useful and the best-studied model system for this enzyme because of their unusually large number of chromosomal ends (17). Holotrichous ciliates (e.g., *Tetrahymena*) contain thousands of macronuclear DNA molecules, and hypotrichous ciliates (e.g., *Euplotes*, which is used in this study) contain millions of gene-sized DNA molecules in their polyploid macronuclei (17). Telomerase is more abundant in these organisms than in other eukaryotes. *Euplotes aediculatus* cells contain approximately 300,000 telomerase molecules per cell (this study) and the activity can easily be detected biochemically.

Numerous ciliate telomerase RNAs have been sequenced, and secondary structure models have been developed (18, 19). These models suggest a common secondary structure for the evolutionarily very distant holotrichous and hypotrichous ciliates. Secondary structure models for telomerase RNAs from other eukaryotes have not yet been derived.

Recently, the purification and cloning of the first telomerase protein subunits, from *T. thermophila*, were reported (20). The two protein subunits are of 95 kDa and 80 kDa molecular mass. The 95-kDa subunit can be crosslinked to substrate oligonucleotides, and the 80-kDa subunit has been shown to bind to telomerase RNA.

Here, we report the purification of telomerase from *E. aediculatus*, a ciliate that is evolutionarily as distant from *Tetrahymena* as plants are from mammals (21). We find polypeptides with apparent molecular masses of 120 kDa and 43 kDa (the latter a doublet) that copurify with telomerase. These two or three putative polypeptide subunits appear to be present stoichiometrically with the RNA. The purified telomerase can extend substrates that contain single-stranded 3' ends, whereas extension of blunt ends was not detectable under the employed reaction conditions. This property of telomerase impacts on models of DNA end replication.

**MATERIALS AND METHODS**

**Growth of Euplotes.** *E. aediculatus* was grown as described (22) under nonsterile conditions with *Chlorogonum* as food source in 15-liter glass jug with aeration.

**Preparation of Nuclear Extracts.** Extracts were prepared as described previously (19) with slight modifications: cells were concentrated with 15 μm Nytext filters and lysed in the presence of 0.8% Nonidet P-40 nonionic detergent. Nuclei were extracted in the presence of 0.4 M potassium glutamate (KGlu); 1 ml of extraction buffer was used per 20 g of original cell pellet.

**Purification of Telomerase.** Nuclear extracts (NXT) were prepared from 45-liter cultures and frozen until a total of 34 ml of extract was obtained. This corresponds to 630 liters of culture (~4 × 10⁶ cells). NXT was diluted to 410 ml to give final concentrations of 20 mM Tris-acetate at pH 7.5, 1 mM MgCl₂, 0.1 mM EDTA, 33 mM KGlu, 10% (vol/vol) glycerol, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride. Diluted NXT was then applied to an Affi-Gel-heparin gel (Bio-Rad) column (230-ml bed volume, 5-cm diameter) equilibrated in the same buffer and eluted with a 2-liter gradient from 33 to 450 mM KGlu.

Abbreviations: NXT, nuclear extract; RNP, ribonucleoprotein particle; snRNP, small nuclear RNP; KGlu, potassium glutamate.

*To whom reprint requests should be addressed.
mM KGl. The column was run at 1 column volume/h at 4°C. Fractions (50 ml) were collected and assayed for telomerase. Telomerase eluted around 170 mM KGl. Fractions containing telomerase (440 ml) were pooled and adjusted to 20 mM Tris-acetate at pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 300 mM KGl, 10% glycerol, 1 mM DTT, 1% Nonidet P-40 (= WB). Per ml of pool, 1.5 nl/ml of each of two competitor DNA oligonucleotides (5'-TAGACCTGTAGTGACATTTGGAAGC-3' and 5'-TAGACCTGTAGTGACATTTGGAAGCTCATCA-3'), 50 μg of yeast RNA, and 0.3 μl of telomerase-specific oligonucleotide [5'-biotin-TAGACCTGTAGTGACATTTGGAAGC-(rmeG)₂-(rmeU)₅-(rmeG)₄-(rmeU)₄-rmeG-3'] were added (rmeN = 2'-O-methylribonucleotides, complementary to the telomerase RNA template region; deoxyribonucleotides were not complementary). This material was added to UltraTrex immobilized Neutravidin Plus (Pierce) column material (60 μl of suspension per ml of pool) that had been preblocked twice for 15 min with WB containing 0.01% Nonidet P-40, 0.5 mg/ml BSA, 0.5 mg/ml lysosome, 0.05 mg/ml glycogen, and 0.1 mg/ml yeast RNA and washed twice with 15 min with WB. These steps were carried out in suspension in a tube on a rotating wheel at 4°C. Column material was centrifuged at 200 x g for 2 min in between to pellet the matrix and to change buffers. The pool—column mixture was incubated 8 min at 30°C and then 2 h at 4°C on a rotating wheel to allow binding. The supernatant containing unbound material was removed after centrifugation and the column material was washed with 10 column volumes each time by shaking, centrifugation, and removal of the supernatant in the following way: column material was rinsed with WB at 4°C, washed 15 min at 4°C with WB, rinsed with WB, washed 5 min at 30°C with WB containing 0.6 M KGl and no Nonidet P-40, washed 5 min at 25°C with WB, and rinsed again with WB. The volume after the final wash was kept small to yield a ratio of buffer to column material of approximately 1:1. Telomerase was eluted by adding 1 nl of displacement deoxyoligonucleotide (5'-CA₆CA₆AC₆TA₆CAG₆TCTA-3') per ml of column material and incubating at 25°C for 30 min. Centrifugation was carried out for 2 min in a microcentrifuge and eluates were collected. The elution step was repeated two more times for 30 min with fresh displacement oligonucleotide each time.

**Native Gel Electrophoresis.** Native (nondenaturing) gels of 3.5% polyacrylamide and 0.33% agarose were poured, and samples were electrophoresed and transferred as described (23). Telomerase comigrated approximately with the xylene cyanol dye.

**Quantitation of Telomerase.** In all fractions most of the telomerase RNA was incorporated into the RNP enzyme, therefore the amount of telomerase was taken as the amount of telomerase RNA (assuming one RNA per RNP). Telomerase RNA was detected by hybridization (19) of molecules in native or denaturing gels that were transferred to nylon membranes (Hybond-N, Amersham). Hybridization was quantitated on a PhosphorImager (Molecular Dynamics). In vitro transcribed telomerase RNA loaded on the same gels served as standard; the signal was proportional to the amount of telomerase RNA loaded.

**T7 Construct and Transcription.** A construct for phage T7 RNA polymerase transcription of *E. aediculatus* telomerase RNA was produced by PCR. The telomerase RNA gene was amplified with primers that annealed to either end of the gene. The primer annealing at the 5’ end also encoded a hammerhead ribozyme sequence to generate the natural 5’ end upon cleavage of the transcribed RNA, a T7-promoter sequence and an EcoRI site for subcloning: 5'-GGCGGAATTCATAC- GACTTACATAGGAGAAACTCTTGAGGGCCGAAAGCGCAATCCGCAAAGTGGAA TTCTCGTATATTAGTCTTAG-3'. The 3’ primer included an EarI site for termination of transcription at the natural 3’ end and a BamHI site for cloning: 5'-GGCGGATCCCTCCTCAAAGATGAGGACAGCAAAC-3’. The PCR amplification product was cleaved with EcoRI and BamHI and subcloned into the respective sites of pUC19 to give pEaT7. The correctness of the insert was confirmed by DNA sequencing. T7 transcription was performed as described (24) with EarI-linearized plasmid. RNA was gel purified and the concentration was determined (an A₂₆₀ of 1 = 40 μg/ml).

**RESULTS**

**Purification of Telomerase.** Telomerase was purified (Table 1) from nuclear extracts of *E. aediculatus*. Telomerase was first enriched by chromatography on an Affi-Gel-heparin column and then purified extensively by affinity purification with an antisense oligonucleotide (Materials and Methods). The method (Fig. 1) was modified from that developed for small nuclear RNP’s (snRNPs) (23, 26). We had isolated the telomerase RNA gene from *E. aediculatus* previously, and found the template region of telomerase RNA to be accessible to hybridization in the telomerase RNP particle (19). Therefore, an antisense oligonucleotide was synthesized complementary to this template region as an affinity bait for telomerase. A biotin residue was included at the 5’ end of the oligonucleotide to immobilize it to an avidin column.

Following binding and extensive washing steps, telomerase was eluted by a novel method involving a displacement oligonucleotide (Fig. 1). The affinity oligonucleotide included DNA bases not complementary to the telomerase RNA 5’ to the telomerase-specific sequence. Because the displacement oligonucleotide is complementary to the affinity oligonucleotide over its entire length, it can form a thermodynamically more stable duplex with the affinity oligonucleotide than does telomerase. As expected, addition of the displacement oligonucleotide to affinity-bound telomerase resulted in efficient elution of telomerase under native conditions (Fig. 1). Telomerase appeared to be approximately 50% pure at this stage as judged by analysis on a protein gel, and only a fraction was purified further on a glycerol gradient (see below).

**Analysis of Telomerase During Purification.** Telomerase was analyzed after each purification step by three separate assays. (i)

<table>
<thead>
<tr>
<th>Table 1. Purification of telomerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Nxt</td>
</tr>
<tr>
<td>Heparin</td>
</tr>
<tr>
<td>Affinity</td>
</tr>
<tr>
<td>Glycerol gradient</td>
</tr>
</tbody>
</table>

Telomerase concentration was measured by native gel electrophoresis and hybridization along with telomerase RNA standards (see Fig 2 and text). Protein concentration in extract and after heparin-column chromatography was measured according to Bradford (25) with BSA standards. NA, not available. *Telomerase was enriched 12-fold in Nxt compared to whole cell extracts with a recovery of 80%; 85% of telomerase was solubilized from nuclei upon extraction. †The 0.3 mg was calculated from the measured amount of telomerase (680 pmol) by assuming a purity of 50% (based on a protein gel).
Telomerase RNA present in fractions was quantified by denaturing gel electrophoresis and Northern analysis. Synthetic T7-transcribed telomerase RNA of known concentration served as standard (see Materials and Methods). The signal of hybridization was proportional to the amount of telomerase RNA, and the derived telomerase RNA concentrations were consistent with but slightly higher than those obtained by native gel electrophoresis. Comparison of the amount of telomerase RNA in whole cell RNA to serial dilutions of known concentrations of the T7 RNA transcript indicated that each *E. aediculatus* cell contains \( \approx 300,000 \) telomerase molecules.

(ii) Native gel electrophoresis was used to analyze the integrity of the telomerase RNP particle (Fig. 2). In nuclear extracts, telomerase was assembled into an RNP particle that migrated slower than unassembled telomerase RNA. Less than 1% free RNA was detectable. Sometimes a slower migrating telomerase RNP complex was also detectable in extracts, but in more recent preparations no such complex was apparent. Upon purification on the heparin column the telomerase RNP particle did not change in mobility. However, upon affinity purification the mobility of the telomerase RNP particle increased slightly (Fig. 2, lane 7, and additional data not shown), suggesting that a protein subunit or fragment had been lost. On glycerol gradients, affinity-purified telomerase did not change in size any further but about 2% free telomerase RNA became detectable (Fig. 2, lane 8), suggesting a small amount of disassembly of the RNP particle.

(iii) Telomerase activity was quantified by assaying the elongation of oligonucleotide primers in the presence of the dGTP and [\( \alpha ^{32} \text{P} \)]dTTP (Fig. 3). The specific activity of telomerase did not change significantly upon purification, and affinity-purified telomerase was fully active. Crude extract (NXT) was diluted 700- to 7000-fold in the assay shown in Fig. 3. At higher concentrations an inhibitory activity was detected in extracts and activity was not linear with concentration. Upon purification this inhibitor was removed, and no inhibitory effect was detected with purified telomerase even at high enzyme concentrations.

**Protein Composition of Purified Telomerase.** Telomerase RNP purified by Affi-Gel-heparin column chromatography, affinity chromatography, and glycerol gradient centrifugation (activity shown in Fig. 3) was analyzed by SDS/PAGE (Fig. 4). Polypeptides with apparent molecular masses of 120 and 43

---

**Fig. 1.** Affinity purification of telomerase (Upper) and elution with a displacement oligonucleotide (Lower). After binding of telomerase to avidin beads via an antisense oligonucleotide containing a biotin residue (bio), telomerase is eluted with a displacement deoxyribonucleotide. Because the displacement oligonucleotide has additional complementarity to the affinity oligonucleotide, it forms a thermodynamically more stable duplex and therefore displaces telomerase from the column. 2'-O-methyl sugars of the affinity oligonucleotide are indicated by the bold line.

**Fig. 2.** Telomerase is maintained as an RNP throughout its purification. Native gel electrophoresis of *in vitro* transcribed telomerase RNA (lanes 1–4) and telomerase RNP from fractions throughout the purification (lanes 5–8). Telomerase was visualized by Northern blot hybridization to its RNA component. Lanes 1–4, 1.5, 4.6, 14, and 41 fmol of telomerase RNA, respectively, lane 5, nuclear extract (containing 42 fmol of telomerase); lane 6, Affi-Gel-heparin-purified telomerase (47 fmol of telomerase); lane 7, affinity-purified telomerase (68 fmol); lane 8, glycerol gradient-purified telomerase (35 fmol). Throughout the paper, telomerase is quantitated by measuring its RNA component.

**Fig. 3.** Telomerase activity is maintained throughout the purification. 5'-(G\( {\text{T}} \)\( {\text{T}} \))\( {\text{G}} \) at 1 \( \mu \text{M} \) was extended in 20-\( \mu \text{l} \) reaction mixtures in the presence of 2 \( \mu \text{l} \) of [\( \alpha ^{32} \text{P} \)]dTTP (10 mCi/ml, 400 Ci/mmol; 1 Ci = 37 GBq) and 125 \( \mu \text{M} \) dGTP as described (19) and loaded on a sequencing gel. Telomerase fractions are labeled as in Fig. 2. Lane 1, no telomerase added; lanes 2, 5, 8, and 11, 0.14 fmol of telomerase; lanes 3, 6, 9, and 12, 0.42 fmol of telomerase; lanes 4, 7, 10, and 13, 1.3 fmol of telomerase. Activity was quantified on a PhosphorImager. Under these conditions, 1 fmol of affinity-purified telomerase incorporated 21 fmol of dTTP in 30 min.
when compared to 37-kDa masses and 43-kDa molecules that ended in 4, and may be an unrelated protein). The 120-kDa species and the doublet at 43 kDa each stained with Coomassie brilliant blue approximately at the level of 1 pmol when compared with the BSA standards. Because this fraction contained 1 pmol of telomerase RNA, all of which was assembled into an RNP particle (Fig. 2, lane 8), there appear to be two polypeptide subunits that are stoichiometric with telomerase RNA. However, it is also possible that the two proteins around 43 kDa are separate subunits of the enzyme.

The 120- and 43-kDa polypeptides also copurified with telomerase when S-Sepharose or Q-Sepharose columns were included in the purification (data not shown) and they comigrated with the telomerase RNP complex in the first dimension of a two-dimensional gel in which the first dimension was nondenaturing and the second dimension was a denaturing SDS/polyacrylamide gel (data not shown). Affinity-purified telomerase that was not subjected to fractionation on a glycerol gradient contained additional polypeptides with apparent molecular masses of 35 and 37 kDa, respectively. This latter fraction was estimated to be at least 50% pure. The 35- and 37-kDa polypeptides present in affinity-purified material were not separated reproducibly by glycerol gradient centrifugation but are suspected to be contaminants since they were not visible in all active preparations.

**Sedimentation Coefficient of Telomerase.** The sedimentation coefficient of telomerase was determined by glycerol gradient centrifugation (Fig. 5). Affinity-purified telomerase cosedimented with catalase at 11.5 S. Telomerase in extracts sedimented slightly faster and peaked around 12.5 S. Therefore, consistent with the mobility on native gels, purified telomerase appears to have lost a proteolytic fragment or a loosely associated subunit.

**Substrate Utilization Influenced by Length and Sequence of the Single-Stranded Overhang.** A simple model for DNA end replication predicts that after semiconservative DNA replication, telomerase extends double-stranded, blunt-ended DNA molecules (Introduction; ref. 8). In a variant of this model, a single-stranded 3' end is created by a helicase or nuclease after replication; this end is then used by telomerase for binding and extension.

To determine whether telomerase can elongate blunt-ended molecules, model hairpins were synthesized with telomeric repeats at their 3' ends. Substrate mobility on a nondenaturing gel indicated very efficient hairpin formation as opposed to dimerization (data not shown). Their substrate efficiencies were compared with double-stranded telomere-like substrates with different length overhangs (Fig. 6). A model substrate that ended with 4 G residues (lanes 1–15) was not elongated when blunt ended (lanes 1–3). Slight extension was detected with an overhang length of 2 bases. Elongation became efficient when the overhang was at least 4 bases long. A double-stranded substrate that ended with 4 T residues behaved similarly, with a 6-base overhang required for highly efficient elongation. The lighter exposure of lanes 25–30 shows a ladder of elongated products with the darkest bands correlating with the putative 5' boundary of the template (19). The abundance of products that correspond to other positions in the template suggests that pausing and/or dissociation occurs at sites other than the site of translocation with this purified telomerase.

Double-stranded blunt-ended oligonucleotides were not substrates for telomerase (Fig. 6). To determine whether these molecules would bind to telomerase, we performed a competition experiment. 5'-end-labeled substrate (GxT)x and a hairpin substrate with a six base-overhang (lanes 25–27, Fig. 6), both at 2 nM, were extended with 0.125 nM telomerase. Whereas the same oligonucleotide substrates unlabeled competed efficiently with labeled substrate for extension, no reduction of activity was observed when the double-stranded blunt-ended hairpin oligonucleotides were used as competitors, even with a 100-fold excess (200 nM) (data not shown).

Therefore, we conclude that double-stranded blunt-ended oligonucleotides cannot bind to telomerase at the concentrations tested; a single-stranded 3' end is required for binding, probably to base pair with the telomerase RNA template.

**Fig. 4.** A 120-kDa polypeptide and a 43-kDa doublet copurify with telomerase. Glycerol gradient fractions were separated on a 4–20% polyacrylamide gel that was stained with Coomassie brilliant blue. Lanes 1 and 2, molecular mass markers, sizes indicated in kDa on the left. Lanes 3–5, glycerol gradient fraction pools: lane 4, pool that contained 1 pmol of telomerase RNA. Lanes 6–9, BSA standards, with the amount of BSA in each lane indicated on the top.

**Fig. 5.** Sedimentation coefficient of telomerase is consistent with an RNP of ~230 kDa. Nuclear extract and affinity-purified telomerase were fractionated on 15–40% glycerol gradients containing 20 mM Tris-acetate at pH 7.5, 1 mM MgCl2, 0.1 mM EDTA, 300 mM KCl, and 1 mM DTT. The telomerase peak was identified by native gel electrophoresis of gradient fractions followed by blot hybridization to its RNA component. Marker proteins were run in a parallel gradient and had sedimentation coefficients of 7.6 S for alcohol dehydrogenase (ADH), 11.3 S for catalase, 17.3 S for apoferritin, and 19.3 S for thyroglobulin. Glycerol gradients were poured in tubes (13 × 51 mm, 5 ml). Centrifugation was performed in a SW55Ti rotor (Beckman) at 55,000 rpm for 14 h at 4°C.
Purified telomerase utilizes double-stranded primers only if they contain a 3′ overhang. Assays were performed with unlabeled 125 μM dGTP, 125 μM dTTP, and 0.02 μM 5′-end-labeled primer in 10-μl reaction mixtures containing 20 mM Tris-acetate at pH 7.5, 10 mM MgCl₂, 50 mM KCl, and 1 mM DTT, at 25°C for 30 min as described (19). Primers (schematically indicated below the Fig.) were incubated without telomerase (−), with 5.9 fmol of affinity-purified telomerase (+), or with 17.6 fmol of affinity-purified telomerase (++).

Lanes 1–15, substrates with telomeric repeats ending with 4 G residues. Lanes 16–30, substrates with telomeric repeats ending with 4 T residues. The putative alignment on the telomerase RNA template (19) is indicated at the top. A lighter exposure of lanes 25–30 is shown on the right to allow visualization of the nucleotides that are added and the positions of pausing in elongated products. Percent of substrate elongated for the third lane in each set was quantified on a Phosphorlmager and is indicated at the bottom. Primer substrates were gel purified, 5′-end-labeled with polynucleotide kinase, heated at 0.4 μM to 80°C for 5 min, and then slowly cooled to room temperature in a heating block to allow renaturation and helix formation of the hairpins. Faint bands below primers in lanes 10–15 that are independent of telomerase represent shorter oligonucleotides in the primer preparations. Sequences of deoxynucleotides were as follows: lanes 1–3, 5′-C₄(A₄C₄)₃CACA(G₄T₄)₃G₄-3′; lanes 4–6, 5′-C₄(A₄C₄)₃CACA(G₄T₄)₃G₄-3′; lanes 7–9, 5′-(A₄C₄)₃CACA(G₄T₄)₃G₄-3′; lanes 10–12, 5′-A₄C₄(A₄C₄)₃CACA(G₄T₄)₃G₄-3′; lanes 13–15, 5′-C₄(A₄C₄)₃CACA(G₄T₄)₃G₄-3′; lanes 16–18, 5′-(A₄C₄)₃CACA(G₄T₄)₃G₄-3′; lanes 19–21, 5′-(A₄C₄)₃CACA(G₄T₄)₃G₄-3′; lanes 22–24, 5′-(A₄C₄)₃CACA(G₄T₄)₃G₄-3′; lanes 25–27, 5′-(A₄C₄)₃CACA(G₄T₄)₃G₄-3′; lanes 28–30, 5′-(A₄C₄)₃CACA(G₄T₄)₃G₄-3′. Affinity-purified telomerase used in the assay was dialyzed with a membrane having a molecular cut-off of 100 kDa to remove the displacement oligonucleotide. Reaction products were separated on an 8% polyacrylamide/urea gel containing 36% formamide to denature the hairpins.
DISCUSSION

We describe the purification of telomerase from E. aediculatus and the identification of two putative protein subunits, 120 and 43 kDa in apparent molecular mass. The 43-kDa subunit was resolved on most gels as a doublet, which could be due to post-translational modifications of this subunit. We consider these proteins to be subunits of telomerase because (i) these proteins copurify with telomerase in several chromatographic systems, on a two-dimensional gel, and on a glycerol gradient and (ii) both are present approximately stoichiometrically with telomerase RNA in the RNP particle. Other proteins in purified fractions were severalfold less abundant as judged by staining and would be subsstoichiometric with telomerase RNA. Verification of any polypeptide as an authentic telomerase subunit will require cloning the gene, expressing recombinant protein, and reconstituting active telomerase from the purified components.

The telomerase protein subunits identified for Tetrahymena are of different size, 80 and 95 kDa (20), but Tetrahymena is an evolutionarily very distant species, so similar sizes of the polypeptides were not expected. The telomerase RNA subunits of Euplotes and Tetrahymena are very different in sequence and size as well (189 and 159 nucleotides, respectively), whereas the secondary structure is conserved (19).

The cornerstone of the purification is the affinity chromatography with an antisense oligonucleotide. We have modified this method (23, 26) by developing a procedure to elute the bound RNP particle under mild conditions with a displacement oligonucleotide. This improvement was crucial for retention of enzyme activity. Furthermore, under the rather mild elution conditions, proteins that bind nonspecifically to the column matrix are not eluted. The method should be applicable for the purification of other RNP particles as well.

Purified telomerase cosediments with catalase on a glycerol gradient at 11.5 S. The calculated molecular mass for telomerase, if it would consist of one 120-kDa protein subunit, one 43-kDa subunit, and one RNA subunit of 66 kDa, adds up to 229 kDa; this is in striking agreement with 223 kDa, the molecular mass of catalase. However, the sedimentation coefficient is a function not only of the molecular mass but also of the partial specific volume and the frictional coefficient of a molecule, both of which are unknown for the telomerase RNP. Therefore, the data only suggest a stoichiometry of 1:1:1 for the protein and RNA subunits.

In a cytological study (27), much of telomerase RNA in both E. aediculatus and Ostyricha nova was localized in macronuclear foci which also contain snRNPs. Here we show that extraction of telomerase from nuclei is very efficient and that close to 100% of telomerase RNA is assembled into active RNP complexes. This suggests that the telomerase RNA observed in the foci is in telomerase-RNP complexes and not just free telomerase RNA.

We tested double-stranded oligonucleotide primers with different length 3’ overhangs in telomerase assays to determine the minimal overhang length and to determine whether telomerase would be able to extend blunt-ended molecules. Blunt-ended molecules are predicted intermediates in DNA end replication in a model in which telomerase acts after semiconservative DNA replication (8). Under the reaction conditions employed, blunt-ended molecules were not substrates, nor were they substrates in nuclear extracts (data not shown). For a substrate ending with 4 T residues (Fig. 6), the extent of elongation increased with overhang length and became very efficient with 6 bases at the 3’ end available for pairing. The substrate set ending with 4 G residues behaved similarly. However, this substrate set was elongated less efficiently and a plateau in activity was reached with an overhang length of only 4 bases. These primer sets are assumed to anneal at two very different positions in the template (Fig. 6, top), which may affect their binding or elongation rate or both.

Studies with partially purified telomerase from Tetrahymena also indicated that double-stranded blunt-ended oligonucleotides were not substrates for the enzyme (28).

A simple explanation for the inability of telomerase to extend completely double-stranded DNA oligonucleotides is provided by the competition experiment. Since the double-stranded blunt-ended oligonucleotides did not compete for binding with active substrate, it appears that a single-stranded 3’ end is required for binding to telomerase, presumably to allow base pairing with the RNA template. Although protein-substrate interactions are thought to occur as well (e.g., see refs. 12 and 20), they must not be sufficient for binding at the concentrations tested. These results suggest that in vivo, if semiconservative DNA replication leaves a blunt-ended chromosome terminus, other factors such as helicases or nucleases must then produce a single-stranded 3’ end to allow telomerase action. Alternatively, telomerase could act before rather than after semiconservative DNA replication on the 3’ overhang of parental telomeres (see model II of ref. 8).

Interestingly, Wellinger et al. (29) found that single-stranded 3’ overhangs can be detected at telomeres late in S phase, even in a yeast strain from which the telomerase RNA gene was deleted. Therefore, these overhangs can be synthesized in the absence of telomerase, perhaps by a 5’→3’ exonuclease acting on an initially blunt-ended intermediate produced by conventional DNA replication (29). Such generated 3’ overhangs could then be extended efficiently by telomerase.

The purification of telomerase from E. aediculatus will now allow thorough characterization of the biochemical behavior of this enzyme and identification of the genes encoding the protein subunits.

We thank Loren Denton for his efforts and success in growing Euplotes cells in very large quantities and for extract preparation, Phil Bevilacqua for the crucial idea to use a displacement oligonucleotide to purify telomerase and discussions, Laura Hendrick for construction of the T7 construct, Cheryl Grosshans for oligonucleotide synthesis, Julie Cooper and Toru Nakamura for discussions, and Julie Cooper, Liz Blackburn, and Raymund Wellinger for critical reading of the manuscript. J.L. was supported by postdoctoral fellowships from the European Molecular Biology Organization and the Swiss National Science Foundation. T.R.C. is an investigator of the Howard Hughes Medical Institute and an American Cancer Society Professor.