Molecular Biology of the Cell
Vol. 14, 4794–4804, December 2003

Tetrahymena Telomerase Is Active as a Monomer

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Submitted July 4, 2003; Revised August 22, 2003; Accepted August 26, 2003
Monitoring Editor: Peter Walter

Telomerase is an enzyme that utilizes an internal RNA molecule as a template for the extension of chromosomal DNA ends. The catalytic core of telomerase consists of the RNA subunit and a protein reverse transcriptase subunit, known as telomerase reverse transcriptase (TERT). It has previously been shown that both yeast and human telomerase can form dimers or multimers in which one RNA in the complex can influence the activity of another. To test the proposal that dimerization might be essential for telomerase activity, we sought to determine whether *Tetrahymena thermophila* telomerase is active as a dimer or a monomer. Recombinant *Tetrahymena* telomerase eluted from a gel filtration column at the size of a monomeric complex (one RNA plus one TERT), and those fractions showed processive telomerase activity. We were unable to detect dimerization of *Tetrahymena* telomerase by coprecipitation experiments, by using tags on either the TERT protein or telomerase RNA. Therefore, a majority, if not all, of the recombinant *Tetrahymena* telomerase in our reconstitution system is present as a monomeric complex. We were also unable to detect dimerization of native telomerase from mating and vegetative *Tetrahymena* cell extracts. These results demonstrate that *Tetrahymena* telomerase does not need to dimerize to be active and processive.

INTRODUCTION

Telomeres form a protective cap on chromosome ends and are usually composed of short G-rich DNA repeats complexed with proteins (reviewed in McEachern *et al*., 2000). The telomeres of unicellular eukaryotes and of the germ cells of multicellular organisms are maintained by the enzyme telomerase, which was first identified in the ciliated protozoan *Tetrahymena thermophila* (Greider and Blackburn, 1985). Telomerase activity has been detected in many human cancer cells and seems to be necessary for their continued growth (Shay and Bacchetti, 1997; Hahn *et al*., 1999; Zhang *et al*., 1999).

The catalytic core of telomerase consists of the RNA subunit (Greider and Blackburn, 1987) and a protein catalytic subunit, known as telomerase reverse transcriptase (TERT) (Lingner *et al*., 1997). Although a complex consisting of TERT and telomerase RNA is sufficient to reconstitute telomerase activity (Weinrich *et al*., 1997; Beattie *et al*., 1998; Masutomi *et al*., 2000; Wenz *et al*., 2001), other components of the telomerase holoenzyme have been identified in various organisms (reviewed in Bryan and Cech, 1999).

The telomerase complex from nuclear extracts of immortal human cells fractionates at ~1000 kDa (Schnapp *et al*., 1998), although this size is reduced to 550–600 kDa upon affinity purification of telomerase, probably reflecting loss of loosely associated subunits (Schnapp *et al*., 1998; Wenz *et al*., 2001). The size of the smaller complex is consistent with being a dimer of two TERT molecules (127 kDa each) and two telomerase RNA molecules (150 kDa each), although this would not leave any room for other telomerase subunits such as hEst1 (160 kDa) (Reichenbach *et al*., 2003; Snow *et al*., 2003). By using an affinity purification technique to measure the number of telomerase RNA molecules in the complex, it was confirmed that baculovirus-expressed recombinant human telomerase does in fact exist as a dimer (Wenz *et al*., 2001). A number of other studies have confirmed that recombinant human telomerase is capable of forming both physical and functional multimers (Armbruster *et al*., 2001; Beattie *et al*., 2001; Arai *et al*., 2002; Moriarty *et al*., 2002). Dimerization of human telomerase may be mediated by a "*trans*-pseudoknot" between two RNA molecules, similar to intermolecular interactions between RNA templates of retroviral reverse transcriptases (Ly *et al*., 2003).

The situation for yeast telomerase is more complex. Prescott and Blackburn (1997) provided convincing evidence for more than one active site in yeast telomerase complexes assembled in vivo). On the other hand, Livengood *et al.* (2002) found nonoverlapping deletions in the telomerase RNA that led to loss of coimmunoprecipitation with Est1p (a yeast telomerase component), or with Est2p (yeast TERT), but not both; these findings are difficult to reconcile with a dimer or multimer, because their yeast strain also contained intact telomerase RNA, which in a dimer (multimer) model would complex with the deleted RNA and lead to substantial coimmunoprecipitation. Similarly, an Est2p mutant protein failed to coprecipitate Est1p and Est3p even in the presence of wild-type Est2p (Friedman *et al*., 2003). Thus, these authors suggested that yeast telomerase is not an obligate multimer in the steady state. This can be reconciled with the identification of multimeric complexes if such complexes occur during specific stages of the cell cycle or certain subcellular locations, such as the telomere.

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03–07–0474. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03–07–0474.

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Abbreviations used: DTT, dithiothreitol; TERT, telomerase reverse transcriptase.

Although the role of telomerase dimerization remains unknown, the fact that one molecule in the subunit can positively or negatively influence the level of activity of the other molecule (Prescott and Blackburn, 1997; Beattie *et al*., 2001; Wenz *et al*., 2001; Ly *et al*., 2003) implies a biological function for the interaction. Several models for the role of telomerase dimerization have been proposed, including the coordinate replication of two telomeres simultaneously, or the switching of primer between two templates to accomplish processive elongation (Wenz *et al*., 2001). Knowledge of the multimerization state of telomerase is therefore fundamental to knowledge of the mechanism of the enzyme.

Telomerase isolated from the ciliate *Euplotes aediculatus* can occur as either a monomer or a dimer (Lingner and Cech, 1996; Aigner *et al*., 2003), whereas *Euplotes crassus* telomerase undergoes multimerization (Wang *et al*., 2002). Gel filtration analysis of telomerase from *Tetrahymena thermophila* showed a complex of ~500 kDa in both vegetative and mated cells (Wang and Blackburn, 1997), which is inconclusive in regards to the multimerization state of telomerase because the protein composition of the complexes was not known. We therefore sought to determine the multimerization state of active recombinant *Tetrahymena* telomerase by using a variety of techniques. We found no evidence for dimerization of *Tetrahymena* telomerase, suggesting that dimerization is not a necessary property of enzymatically active telomerase.

MATERIALS AND METHODS

Plasmids

The construction of a synthetic Tt_TERT gene has been described previously (Bryan *et al*., 2000). This gene was inserted into the vector pET-28a (Novagen, Madison, WI) to give the construct pET-28a-TERT, encoding *Tetrahymena* TERT with N-terminal T7 and 6 × His tags (Bryan *et al*., 2000). This plasmid was modified to give pFLAG-TERT by insertion of oligonucleotides encoding a FLAG tag in place of the N-terminal T7 and 6 × His tags.

A plasmid containing the *Tetrahymena* telomerase RNA gene, a promoter for T7 RNA polymerase and a hammerhead ribozyme self-cleavage domain to process the 5′ end of the RNA has been described and called pTET-telo (Zaug and Cech, 1995). Polymerase chain reaction (PCR) mutagenesis was used to make a mutant version of pTET-telo, with a C-to-U mutation at position 48 of the telomerase RNA (pTET-telo-48U); this plasmid was a gift from Art Zaug. A plasmid containing tandem *Tetrahymena* telomerase RNA genes separated by a 12-nt linker was constructed by PCR amplification of the gene from pTET-telo-48U (with primers tb115 [5′-CGCGGATCCATACCCGCTTAATTCATTCAG] and tb116 [5′-AAAACTGCAGGCTCTTCCAAAATAAGACATC]) followed by its insertion into the *Bam*HI and *Pst*I sites of pTET-telo, downstream of the existing telomerase RNA gene. This construct (pTET-telo-48U-3′) contains a C48U mutation in the 3′ half of the double RNA. A second plasmid (pTET-telo-48U-5′) containing a 48U mutation in the 5′ half of the double RNA was constructed in an analogous manner.

A plasmid (phTR+hh) designed for in vitro transcription of human telomerase RNA (hTR) was a gift from Jamie Sperger (University of Colorado, Boulder, CO). The hTR gene was amplified from a previously described pGEM-based construct (Bryan *et al*., 1997) and inserted into pUC19 downstream of a promoter for T7 RNA polymerase and a hammerhead ribozyme self-cleavage domain to process the 5′ end of the RNA.

A version of pTET-telo containing a two-nucleotide nontemplate mutation in the *Tetrahymena* telomerase RNA (AU65–66CC) was a gift from Jamie Sperger. Versions of this plasmid and the wild-type pTET-telo lacking the hammerhead sequence and the first 20 nt of the telomerase RNA were constructed [called pTET-telo21-159(65/66) and pTET-telo21-159 respectively]. They were constructed by PCR from the respective template plasmid with primers tb111 (5′-CAGTGAATTCTAATACGACTCACTATAGATCTGTAATAGAACTGTCA) and ttmut6 (5′-GCAGTGAGCGCAACGCAATTAATG), digestion of the PCR products with *Eco*RI and *Bam*HI and ligation into *Eco*RI/*Bam*HI digested pUC19.

Translation and Reconstitution of Tetrahymena Telomerase

Tetrahymena telomerase RNA was transcribed in vitro from the plasmids pTET-telo, pTET-telo-48U-5′ or pTET-telo-48U-3′ as described previously (Bryan *et al*., 2000), except that the latter two plasmids were linearized with *Sap*I before transcription rather than *Ear*I. All RNAs were gel-purified before inclusion in in vitro translation reactions using pET-28a-TERT to translate *Tetrahymena* TERT protein. The translations were carried out using the TnT; rabbit reticulocyte lysate system (Promega, Madison, WI), as per the manufacturer's directions and as described previously (Bryan *et al*., 2000). The reactions included 20 ng/μl plasmid DNA, 0.8 μCi/μl [^{35}S]methionine, and 20 nM (wild-type *Tetrahymena*) or 200 nM (*Tetrahymena* double RNA) telomerase RNA. The amount of TERT protein produced was estimated by electrophoresis of a portion of the reaction on an SDS-PAGE gel and scintillation counting of the excised protein band in comparison with [^{35}S]methionine standards (Donato *et al*., 1988). We estimated yields of ~20 nM of full-length TERT. Although the concentration of telomerase RNA added to the reaction was equal to or greater than the amount of TERT translated, a large proportion of the TERT was apparently not bound to RNA (Figure 1B). This is presumably due to degradation of a portion of the telomerase RNA in the translation reaction.

Gel Filtration

Reticulocyte lysate translation reactions (600 μl) were either loaded on a Sephacryl gel filtration column directly or treated in one of three ways before loading: 1) buffer exchanged on a Microcon 100 filter unit (Millipore, Bedford, MA) by three rounds of addition of 200 μl of TMG-150 buffer (10 mM Tris acetate, pH 8.0, 1 mM $MgCl_2$, 10% glycerol, 150 mM potassium glutamate) followed by centrifugation at 2500 × *g* for 30 min at 4°C, resulting in recovery of ~800 μl of sample; 2) buffer exchanged on a BioSpin P6 column (Bio-Rad, Hercules, CA) by washing the column three times in TMG-150 followed by centrifugation of the sample on the column at 1000 × *g* for 4 min at 4°C; or 3) filtration through a 0.2-μm syringe filter. No differences were seen in subsequent elution patterns after these different treatments. Gel filtration was performed on a Sephacryl S-300 16/60 column (120-ml bed volume) on a fast-performance liquid chromatography machine (Amersham Biosciences, Piscataway, NJ). The column was run at 0.25 ml/min in TMG-150 at 4°C and 2-ml fractions were collected. Fractions were flash frozen in liquid nitrogen and stored at −80°C. Aliquots (20 μl) were electrophoresed on 4–20% acrylamide SDS gels (Novex, San Diego, CA) that were analyzed with a Typhoon PhosphorImager (Molecular Dynamics, Sunnyvale, CA) to detect ^{35}S-labeled TERT protein, and the bands quantitated with ImageQuant (Molecular Dynamics). Gel filtration standards (Amersham Biosciences) were run separately on the same column under the same conditions, at concentrations of 6 mg/ml (thyroglobulin, catalase, aldolase, and albumin) or 0.6 mg/ml (ferritin). The parameter K_{av} was calculated for each standard using the following formula:

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where V_e is elution volume, V_0 is void volume of column (40 ml) and V_t is total volume of column (120 ml). A standard curve was obtained by plotting K_{av} versus the logarithm of molecular mass and used for estimation of the molecular mass of eluted samples.

Northern Blots

Gel filtration fractions (20 μl) or input translation reaction (1 μl) was added to 10 μl of loading buffer (0.05% bromphenol blue, 0.05% xylene cyanol in formamide), heated to 70°C for 10 min, loaded on a 1.5-mm-thick 4% polyacrylamide/8 M urea gel and electrophoresed at 35 W for 1 h. Radiolabeled size markers were generated by transcription of Century Plus markers (Ambion, Austin, TX) in the presence of [α-^{32}P]CTP and 10^4 cpm were loaded on the gel. The gel was transferred to Hybond-N^+ nylon membrane (Amersham Biosciences) on an electroblotter (Hoeffer, San Francisco, CA) in 0.5× Tris borate-EDTA (45 mM Tris, 45 mM boric acid, 1 mM EDTA) at 0.5 A for 16 h. The membrane was incubated in 10 ml of Church buffer (Church and Gilbert, 1985) at 55°C for 30 min, followed by addition of 5 × 10^6 cpm of ^{32}P-labeled oligonucleotide probe and incubation for 16 h at 55°C. The membrane was then washed three times for 10 min at room temperature in 0.1× SSC/0.1% SDS (15 mM sodium chloride, 1.5 mM sodium citrate, 0.1% SDS). The probe used was Elu3b (5′-TATCAGCACTAGATTTTTGGGGTTGAATG), against *Tetrahymena* telomerase RNA. The membrane was analyzed with a PhosphorImager, and the bands were quantitated with ImageQuant.

Telomerase Activity Assays

Recombinant *Tetrahymena* telomerase activity was measured by incubating 24 μl of gel filtration fractions or 1 μl of column input in a 30-μl reaction including 1× telomerase buffer (50 mM Tris-Cl, pH 8.3, 1.25 mM $MgCl_2$, 5 mM dithiothreitol [DTT]), 1 μM primer [$(GGGGTT)_3$], 100 μM dTTP, and 10 μM [α-^{32}P]dGTP at 80 Ci/mmol (0.1 μl of nonradioactive dGTP at 2.7 mM and 2.4 μl of [α-^{32}P]dGTP at 10 mCi/ml, 800 Ci/mmol; PerkinElmer Life Sciences, Boston, MA). The reaction was incubated at 30°C for 60 min and then processed and electrophoresed on a 12% polyacrylamide/8M urea gel as described previously (Bryan *et al*., 2000). A 100-mer DNA oligonucleotide was radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and 5000 cpm added to the reaction before phenol/chloroform extraction as a recovery

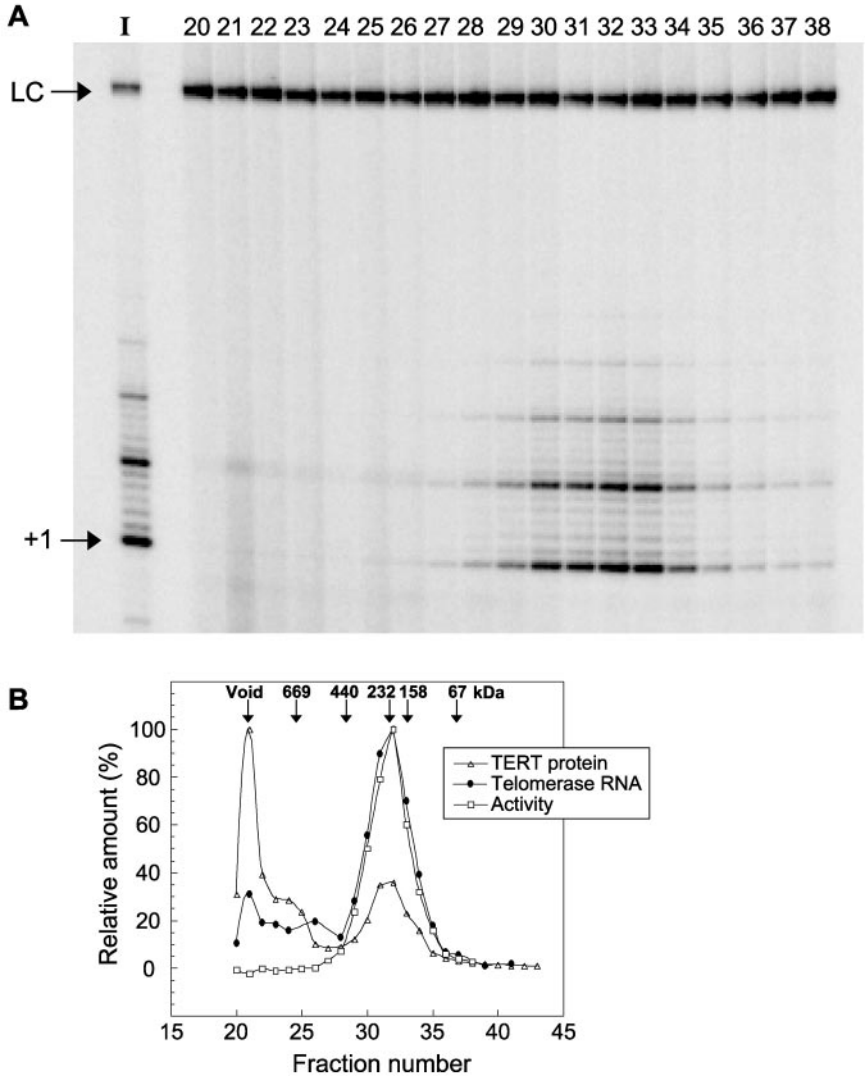

Figure 1. Gel filtration analysis of the size of recombinant *Tetrahymena* telomerase. TERT protein was translated in rabbit reticulocyte lysate in the presence of telomerase RNA, and run over a Sephacryl S-300 column in 150 mM potassium glutamate. (A) The fractions eluting from the column were assayed for in vitro telomerase activity. Lane I, column input. LC, ^{32}P-labeled 100-mer DNA loading control. +1, first telomerase extension product. (B) Telomerase activity (from the gel in A), levels of telomerase RNA (from a Northern blot) and TERT protein (from an SDS-PAGE gel) of the column fractions were quantitated and expressed relative to the fraction with the highest level. The elution profile of protein standards run on the same column is indicated at the top of the graph: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and albumin (67 kDa). The void volume contains proteins of >1500 kDa.

control. Activity from *Tetrahymena* extracts was assayed in the same way with two exceptions: 1) the final dGTP concentration in the reaction was 1.25 μM with a specific activity of 800 Ci/mmol, and 2) the internal DNA standard was 12 nt in length.

Tetrahymena Extract Preparations

T. thermophila strains B2086.1 and CU428.2 were gifts from Peter Bruns and Donna Cassidy-Hanley (Cornell University, Ithaca, NY). Cells were grown vegetatively or starved then mated, and harvested as described previously (Bryan *et al*., 1998). S100 extracts were made from the cells as described previously (Greider and Blackburn, 1987). The extracts were partially purified on a 2 ml DEAE agarose (Bio-Rad) column. The column was equilibrated with TMG (10 mM Tris acetate, pH 8.0, 1 mM $MgCl_2$, 10% glycerol) before loading 5 ml of S100 extract, washed with 6 ml of TMG-200 (TMG plus 200 mM potassium glutamate), and eluted with 12 ml of TMG-350 in 1-ml fractions. Fractions containing active telomerase were pooled, flash frozen, and stored at −80°C. Partially purified extract (430 μl) was diluted to a final potassium glutamate concentration of 150 mM with TMG before loading on a Sephacryl S-300 column (see above), or dialyzed for 3 h into TMG at 4°C before use in a telomerase activity assay (see below).

Immunoprecipitations

FLAG-tagged and T7-tagged TERT were translated in separate reticulocyte lysate translation reactions (200-μl total volume for each reaction), including 4 μg of pFLAG-TERT or pET-28a-TERT, respectively, 200 nM telomerase RNA, and other components as specified by the manufacturer (see above). Two translations were carried out for each protein, one containing 160 μCi of [^{35}S]methionine and 4 μl of "amino acid mixture minus methionine" (provided in the kit), and the other with no [^{35}S]methionine, 4 μl of amino acid mixture minus methionine, and 4 μl of "amino acid mixture minus leucine." After 60 min at 30°C, puromycin was added to a final concentration of 5 μg/ml to stop translation. An aliquot (70 μl) of FLAG-tagged TERT + ^{35}S was mixed with an equal volume of T7-tagged TERT − ^{35}S, and incubated at 30°C for 30–60 min. The opposite combination (T7-tagged + ^{35}S, FLAG-tagged − ^{35}S) was also mixed and incubated. Each of these mixtures, as well as the individual proteins, was immunoprecipitated with either T7-tag antibody-agarose beads (Novagen) or anti-FLAG M2 affinity agarose beads (Sigma-Aldrich, St. Louis, MO). Beads (50 μl) were washed four times in 750 μl of wash buffer-50 (20 mM Tris acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM DTT, 50 mM potassium glutamate), centrifuging at 1500 × *g* for 2 min between washes. The beads were incubated twice with 500 μl of blocking buffer (wash buffer-50 plus 0.5 mg/ml lysozyme, 0.5 mg/ml bovine serum albumin, 0.05 mg/ml glycogen, 0.1 mg/ml yeast RNA) for 15 min at 4°C with agitation. Reticulocyte lysate translation reaction or mixture (50 μl) was added to 50 μl of blocking buffer and centrifuged at 16,000 × *g* for 10 min at 4°C to remove any particulates. The supernatant from that spin was added to 12.5 μl of blocked beads and agitated at 4°C for 2 h. The beads were washed four times in 185 μl of wash buffer-50 and resuspended in 12.5 μl of wash buffer-50 to make a 1:1 slurry. Aliquots of the bead slurry (5 μl), supernatant (5 μl) or original translation reaction (2.5 μl) were added to Laemmli's sample buffer (125 mM Tris-Cl, pH 6.8, 4% SDS, 0.005% bromphenol blue, 20% glycerol, 0.72 M β-mercaptoethanol), heated to 100°C for 3 min, and electrophoresed in a 4–20% polyacrylamide/SDS gel. The gel was fixed in 25% isopropyl alcohol, 10% acetic acid for 30 min, dried at 80°C, and exposed to a PhosphorImager screen overnight. The experiment shown in

Figure 3 used the above-mentioned conditions; variations of this experiment were telomerase RNA concentrations of 20 and 80 nM; blocking buffer and wash buffer potassium glutamate concentrations of 100 and 300 mM; and inclusion of 1 μM $(GGGGTT)_3$ DNA primer at the mixing, immunoprecipitation, and wash steps.

Biotinylated RNA Pulldowns

Biotinylated versions of wild-type and mutant (AU65–66CC) *Tetrahymena* telomerase RNA were constructed by splint ligation. The plasmids pTET-telo21-159 and pTET-telo21-159(65/66) were transcribed in vitro to produce telomerase RNAs lacking the first 20 nt. Transcription was carried out as described previously (Bryan *et al*., 2000) with the exception that the extra $MgCl_2$ step was omitted (because these constructs do not contain hammerhead sequences), and GMP was added to a final concentration of 10 mM. The latter modification resulted in the presence of 10 times more GMP than GTP in the reaction, which would result in most of the 5′ ends of the transcript having a monophosphate, enabling subsequent ligation. An RNA oligonucleotide representing the first 20 nt of telomerase RNA with a 5′ biotin moiety was synthesized on a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA) and gel purified on a 20% polyacrylamide/8M urea gel. This oligonucleotide, the RNA transcript and a DNA oligonucleotide complementary to the first 40 nt of telomerase RNA (the "DNA splint") were combined (400 pmol of each) and ethanol precipitated. The pellet was resuspended in 16 μl of TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA), heated to 90°C for 3 min, and cooled slowly to 30°C over 1.5 h. The reaction was incubated on ice for 5 min before the addition of 2 μl of 10× ligase buffer (500 mM Tris-Cl, pH 8.2, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, 0.5% Triton X-100) and 2 μl of T4 DNA ligase. The ligation reaction was submerged in a 37°C water bath for 4 h. The ligated RNA product was gel purified on an 8% polyacrylamide/8 M urea gel.

A reticulocyte lysate translation reaction was carried out (see above) by using pET-28a-TERT as a template and no telomerase RNA. Biotinylated and nonbiotinylated wild-type and mutant telomerase RNAs (5 pmol) was added to 50-μl aliquots of the translation reaction either singly or together, and incubated at 30°C for 15 min. The biotinylated RNA was precipitated from the reaction on NeutrAvidin agarose beads (Pierce Chemical, Rockford, IL) as follows. Beads (120 μl) were washed four times in 1.8 ml of wash buffer-100 (see "Immunoprecipitations" above; contains 100 mM potassium glutamate), centrifuging at 1500 × *g* for 2 min between washes. The beads were incubated twice with 1.2 ml of blocking buffer (see above; contains 100 mM potassium glutamate) for 15 min at 4°C with agitation. Reticulocyte lysate translation reaction with RNA (40 μl) was added to 40 μl of blocking buffer and centrifuged at 16,000 × *g* for 10 min at 4°C to remove any particulates. The supernatant from that spin was added to 20 μl of blocked beads and agitated at 4°C for 1 h. The beads were washed four times in 260 μl of wash buffer-300 (contains 300 mM potassium glutamate) and resuspended in 20 μl of wash buffer-300 to make a 1:1 slurry. Aliquots of the bead slurry (10 μl), supernatant (20 μl), or original translation reaction (10 μl) were added to 90 μl of ProK buffer (10 mM Tris-Cl, pH 7.5, 7.5 mM EDTA, 0.6% SDS, 50 μg/ml proteinase K) and incubated at 45°C for 30 min then at 95°C for 5 min to release the RNA from the NeutrAvidin beads. The reactions were phenol/chloroform extracted (pH 8.0) and ethanol precipitated before analysis by Northern blotting (see above). A ^{32}P-labeled 100-mer DNA oligonucleotide (5000 cpm) was added to the reactions before phenol/chloroform extraction as a recovery control. The probes used for Northern blotting were tb117 (5′-GAAGGTTATATCAGCACTAG) for wild-type telomerase RNA and tb113 (5′-GAAGGTTATGGCAGCACTAG) for mutant AU65–66CC.

Biotinylated DNA Pulldowns

The telomeric DNA oligonucleotides used were Tet3 $[(GGGGTT)_2]$ and Bio-Tet1 [Biotin-$(GGGGTT)_3$]. To eliminate suspected G-quartet structure, nontelomeric oligonucleotides Tet13A $[TT(GAGGTT)_3]$ and Bio-Tet33A [Biotin-$TT(GAGGTT)_4$] were used in subsequent experiments. Reticulocyte lysate translated telomerase (12 μl) and partially purified (DEAE) *Tetrahymena* extract (20 μl) were incubated for 5–30 min at 30°C with telomeric (1 μM) or nontelomeric (5 μM) primers, 1× telomerase buffer (see above), 100 μM ddTTP, and [α-^{32}P]dGTP at 10 μM and 80 Ci/mmol (recombinant telomerase) or 1.25 μM and 800 Ci/mmol (extracts) in a total volume of 80 μl. Half of each reaction was extracted once with phenol/chloroform/isoamyl alcohol 25:24:1 and once with chloroform/isoamyl alcohol 24:1, whereas the other half remained on ice. An aliquot of each (1/4) was removed as the "input" and the remainder was added to 10 μl of washed and blocked NeutrAvidin beads (see "Immunoprecipitations" for details, except that concentrations of blocking reagents were increased to 0.75 mg/ml (lysozyme and bovine serum albumin) and 0.15 mg/ml (glycogen and yeast RNA), a nonspecific oligonucleotide [PBR(24): 5′-AGCCACTATCGACTACGCGATCAT] was added to a concentration of 5 μM and salt concentrations varied from 100 to 400 mM potassium glutamate). Pulldowns were incubated on ice for 15 min and then washed four times in 100 μl of wash buffer (see above) at 23°C. TES (50 mM Tris-Cl, pH 8, 20 mM EDTA, 0.2% SDS) was added to input, supernatant, and beads fractions to a final volume of 100 μl, reactions were heated to 95°C for 5 min, phenol/chloroform extracted, and ethanol precipitated. A ^{32}P-labeled 100-mer DNA oligonucleotide (5000 cpm) was added to the reactions before phenol/chloroform extraction as a recovery control. For reactions with extracts, half of each reaction was electrophoresed on a 12% polyacrylamide/urea sequencing gel and half was subjected to Northern blotting for telomerase RNA. For recombinant telomerase, half of each reaction was removed before TES addition and electrophoresed on an SDS-PAGE gel, and the remainder was electrophoresed on a 12% polyacrylamide/urea sequencing gel.

Native gel electrophoresis of telomerase RNA dimers

Telomerase RNAs from human and *Tetrahymena* were in vitro transcribed from the plasmids pTet-telo and phTR+hh that had been linearized with Ear1 and Fok1, respectively (Bryan *et al*., 2000), and gel-purified. Dimerization reactions were performed essentially as described previously (Ly *et al*., 2003). Unlabeled RNA (6.7 pmol) was combined with 10^4 cpm of end-labeled RNA in water and denatured at 95°C for 3 min. The reactions were snap-cooled on ice, adjusted to 25 mM Tris-Cl, pH 7.0, 50 mM NaCl and either 10 or 1.25 mM $MgCl_2$, and then either kept on ice (−) or prewarmed at 37°C or 30°C (+) for 2 h before electrophoresis on a 5% acrylamide nondenaturing gel in 90 mM Tris-borate, 1 mM $MgCl_2$ at 10 W for 1 h with no cooling.

RESULTS

Recombinant Tetrahymena Telomerase Is the Size of a Monomer

We reconstituted active *Tetrahymena* telomerase by translation of ^{35}S-labeled TERT protein in rabbit reticulocyte lysate in the presence of in vitro-transcribed *Tetrahymena* telomerase RNA. The size of the reconstituted telomerase complex was analyzed by gel filtration on a Sephacryl S-300 column. The fractions were assayed for in vitro telomerase activity (Figure 1A) and gave a pattern of extension products with a six-nucleotide periodicity that was previously shown to represent processive elongation of the DNA primer (Bryan *et al*., 2000). The same fractions were run on SDS-PAGE gels to determine the elution profile of the ^{35}S-labeled TERT protein, and on a Northern blot to detect the telomerase RNA (Figure 1B).

Telomerase activity eluted reproducibly with a peak in column fractions 31–33. Most of the TERT protein eluted in the void volume (representing complexes of >1500 kDa), but there was also a protein peak in fractions 31–33. Most of the telomerase RNA also eluted in fractions 31–33. The TERT protein in the void showed no telomerase activity, consistent with there being only a small amount of telomerase RNA in this fraction. The amount of TERT protein in the void relative to that in fractions 31–33 decreased as the amount of telomerase RNA in the reaction was increased, and TERT protein translated in the absence of telomerase RNA eluted completely in the void volume (our unpublished data). Thus, it seems that TERT not in a complex with telomerase RNA exists in large aggregates, perhaps consisting of misfolded protein together with chaperones or other proteins.

The elution profile of telomerase was compared with that of proteins of known molecular mass run on the same column (Figure 1B). Fractions 31–33 correspond to a molecular mass of ~180–220 kDa. TERT protein (with T7 and His tags) is ~137 kDa and telomerase RNA is ~52 kDa, so a complex consisting of one TERT plus one RNA would be ~189 kDa, whereas a dimer would be 378 kDa. The elution profile of active *Tetrahymena* telomerase is thus most consistent with it being a monomeric complex of one TERT with one RNA.

The above-mentioned results were reproduced in seven separate runs of the Sephacryl column. The reticulocyte lysate translation reactions were untreated or treated in several different ways to remove unincorporated [^{35}S]methionine and/or aggregated material before running of the column. Treatments included filtration on a 0.2-μm filter

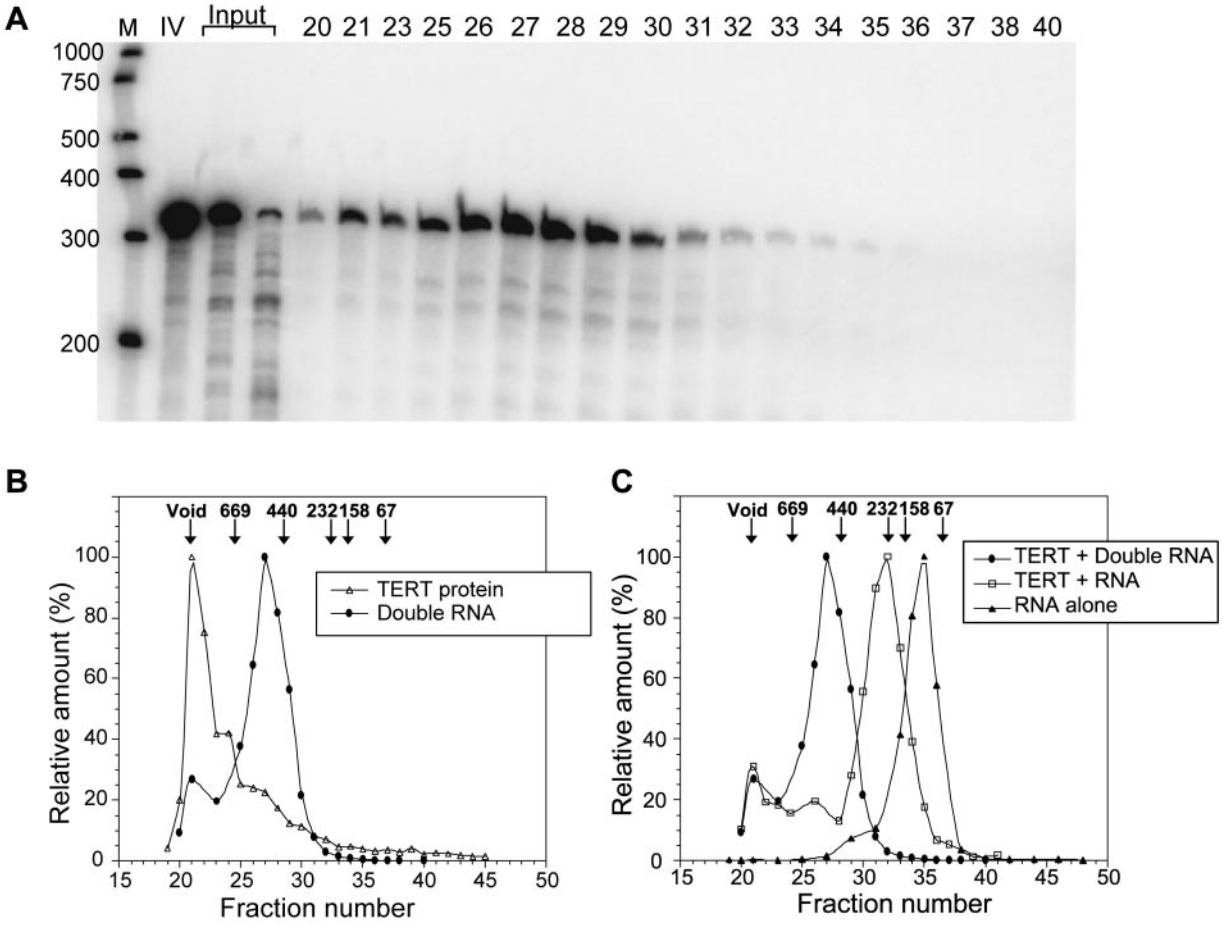

Figure 2. Gel filtration analysis of an artificial *Tetrahymena* telomerase dimer. TERT protein was translated in the presence of an RNA construct consisting of two copies of telomerase RNA (double RNA) and run on a Sephacryl S-300 column. (A) Northern blot of the indicated column fractions, with a telomerase RNA probe. IV, in vitro-transcribed double RNA. Labeled RNA size markers are shown on the left (with size in nucleotides). (B) Quantitation of levels of RNA (from A) and levels of TERT protein in the same fractions (from an SDS-PAGE gel). The elution pattern of size standards is shown (see Figure 1 legend for identities of standards). (C) Comparison of the elution profiles of double RNA in the presence of TERT (from A), wild-type telomerase RNA in the presence of TERT (from Figure 1), and wild-type telomerase RNA alone.

and size exclusion on either a Microcon 100 or a BioSpin P6 column. The column was also run with translation reaction that had been concentrated fourfold on a Microcon 100 column (to a final TERT concentration of ~80 nM), in case dimerization of telomerase is concentration-dependent, with no change in the results. Increasing the amount of telomerase RNA 10-fold (to 200 nM) also did not change the apparent molecular mass of the complex.

An Artificial Telomerase Dimer Elutes at Approximately Its Expected Mass

Because the proteins used as size markers were globular proteins rather than ribonucleoproteins, it seemed possible that the elution profile of telomerase did not represent its true mass. We therefore constructed a control molecule that should mimic a *Tetrahymena* telomerase dimer. We made a construct that encodes two tandem telomerase RNAs, separated by a 12-nucleotide linker (called "double RNA"). A mutation in the template region (C48U) was incorporated into one of the RNAs to be able to distinguish activity arising from either half of the molecule. Two constructs were made, one with C48U in the most 5′ RNA, and one with this mutation in the 3′ RNA. Each of these RNAs was in vitro transcribed and included in a reticulocyte lysate translation reaction with TERT. The C48U mutation results in just one band in a standard telomerase activity assay, as opposed to the processive reaction seen with wild-type RNA. Telomerase assays on each of the double RNA constructs showed that the wild-type template was active regardless of its position in the double RNA molecule (our unpublished data). Activity was only ~4% of that obtained with wild-type telomerase RNA, indicating that only a small proportion of the double RNA molecule formed a complex with one or more TERT molecules and/or that the conformation of the double RNA interfered with its normal function.

The double RNA complex with the C48U mutation in the 5′ half was run on a Sephacryl S-300 column. Telomerase activity was too weak to be detected in the column fractions, and most of the TERT protein was in the void volume (Figure 2B). However, a Northern blot showed that the RNA eluted with a peak in fraction 27 (Figure 2, A and B), corresponding to a molecular mass of ~460–550 kDa. This is only

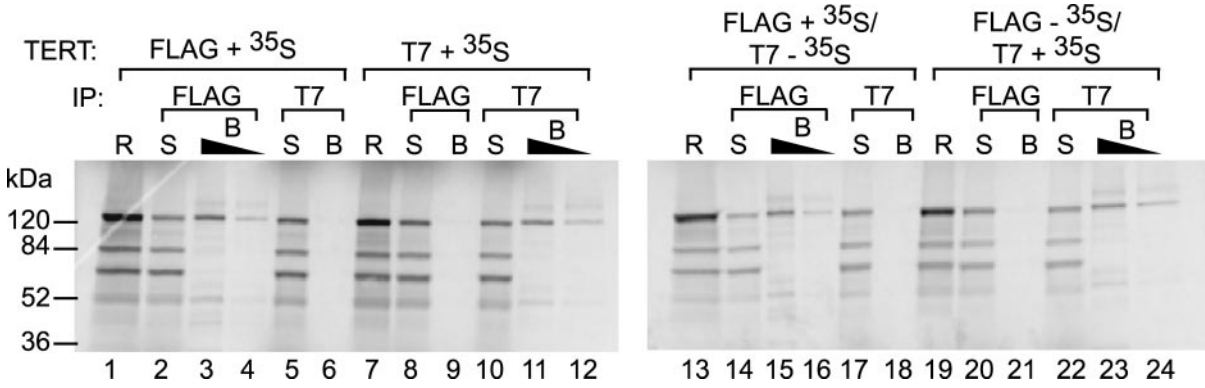

Figure 3. No coimmunoprecipitation of two differentially tagged *Tetrahymena* TERT proteins. FLAG-tagged or T7-tagged TERT was translated in rabbit reticulocyte lysate in the presence of telomerase RNA, either with or without [^{35}S]methionine. Each translation reaction was then assayed separately (left) or mixed in the presence of puromycin and incubated for 15 min at 30°C (right). The translation reactions were immunoprecipitated with FLAG antibody beads or T7 antibody beads (IP). Aliquots of the reticulocyte lysate translation reaction (R), supernatant (S), and beads (B) were loaded on an SDS-PAGE gel. A 1:5 dilution of the beads was also loaded (indicated by triangle above lane). Protein size markers are indicated on the left.

slightly larger than one would expect for the size of the RNA molecule and two TERT molecules (378 kDa). This suggests that the telomerase ribonucleoprotein does not run at a vastly aberrant size on this column.

As a further control for the mass of molecules in different fractions of the column, we ran in vitro-transcribed telomerase RNA alone (in the absence of both TERT and reticulocyte lysate). This RNA peaked in fraction 35, corresponding to a molecular mass of 105–125 kDa (Figure 2C). Because telomerase RNA has a molecular mass of ~52 kDa, it elutes at a higher mass from the column than expected. However, this result and that of the double RNA described above suggest that telomerase RNPs elute at slightly higher masses than expected, so it is unlikely that the apparent mass of 180–220 kDa for wild-type telomerase is an underestimate.

We also ran partially purified extract from vegetatively growing *Tetrahymena* on the Sephacryl column. Telomerase activity eluted in fraction 27, at ~460–550 kDa (our unpublished data). This is consistent with results others have obtained (Greider and Blackburn, 1987; Wang and Blackburn, 1997) and could indicate that *Tetrahymena* telomerase exists as a dimer in vivo but is equally consistent with it being a monomer with other proteins present in the complex.

Coimmunoprecipitation of Tagged Tetrahymena Telomerase Detects Only One TERT per Complex

Coimmunoprecipitation of differentially tagged TERT molecules from human and *E. crassus* has provided evidence that these proteins form multimers (Beattie *et al*., 2001; Armbruster *et al*., 2001; Moriarty *et al*., 2002; Wang *et al*., 2002). We therefore used this technique to determine whether *Tetrahymena* TERT multimerizes. TERT proteins with two different N-terminal tags (FLAG or T7) were expressed separately in reticulocyte lysates in the presence of telomerase RNA, either with or without [^{35}S]methionine. Puromycin was then added to the reaction to terminate translation (and hence prevent further incorporation of ^{35}S). FLAG-tagged labeled protein was mixed with T7-tagged unlabeled protein, and vice versa. Immunoprecipitation was carried out by using immobilized FLAG or T7 antibodies, and aliquots of the input, supernatant, and antibody beads were electrophoresed on SDS-PAGE gels to visualize labeled TERT protein (Figure 3).

The left panel in Figure 3 shows each of the labeled proteins alone as a control, demonstrating that no detectable FLAG-TERT is precipitated by T7 beads and vice versa (lanes 6 and 9). The right panel of Figure 3 shows immunoprecipitation of the mixed proteins. No labeled FLAG-TERT is detectable on T7 beads and vice versa (lanes 18 and 21). Thus, we are not able to detect multimerization of *Tetrahymena* TERT. Each of the positive control beads was also loaded on the gel as a 1:5 dilution to demonstrate that as little as one-fifth the amount of labeled protein would still be detectable (lanes 4, 12, 16 and 24). If all of the TERT protein existed in dimeric complexes, the amount of labeled FLAG-TERT pulled down on T7 beads would be half the intensity of that pulled down on FLAG beads.

The conditions used to attempt to promote dimerization in this experiment (i.e., separate translation followed by incubation at 30°C for 15–60 min) could conceivably be ineffective; however, we also carried out experiments in which the two proteins were cotranslated in the same reaction, followed by coimmunoprecipitation and detection on Western blots, and no dimerization was detectable (our unpublished data).

The experiments in Figure 3 were repeated under several different experimental conditions in an effort to encourage dimerization. The concentration of potassium glutamate used during immunoprecipitation and washing was varied from 50 to 300 mM. Telomerase RNA was included at concentrations of 20 to 200 nM, and the immunoprecipitation was carried out in the presence or absence of 1 μM telomeric DNA primer. The results of all of these experiments were identical to those shown in Figure 3.

Pulldown of Biotinylated Tetrahymena Telomerase RNA Molecules Detects Only One RNA per Complex

Results from the coimmunoprecipitation experiments described above made it unlikely that there was more than one TERT molecule in the active complex. However, the resolution of the gel filtration column (Figure 1) was not fine enough to be able to rule out the existence of a complex of one TERT molecule with two telomerase RNA molecules. We therefore devised a strategy to detect dimerization of the RNA.

Telomerase RNA was biotinylated at its 5′ end, mixed with nonbiotinylated telomerase RNA, reconstituted into a complex with reticulocyte lysate-translated TERT, and isolated by affinity purification on NeutrAvidin beads. Telomerase activity assays confirmed that the biotinylated RNAs supported telomerase activity (our unpublished data). The RNA used was either wild-type or had a mutation incorporated at nontemplate positions 65 and 66 (AU to CC) to be able to distinguish mutant from wild-type on a Northern blot. The probes used for wild-type or mutant RNA were completely specific (our unpublished data). Biotinylated wild-type and mutant RNA were efficiently isolated on the beads (Figure 4, A and B, lanes 3 and 17) with very little background binding of nonbiotinylated RNA (lanes 7 and 21). When biotinylated mutant RNA was reconstituted with TERT in the presence of nonbiotinylated wild-type RNA, no wild-type RNA was detectable after affinity purification (Figure 4A, lane 14). The converse was also true (Figure 4B, lane 24). Again, the RNA on positive control beads was readily detectable when loaded at a fivefold dilution (lanes 4, 11, 18, and 28). An SDS-PAGE gel of ^{35}S-labeled TERT protein (Figure 4C) demonstrated that the protein was not lost during affinity purification of the RNA; it also confirmed that affinity purification of the complex was dependent on having biotin on one or the other of the RNAs. Thus, we were able to detect the presence of only one telomerase RNA molecule per telomerase complex.

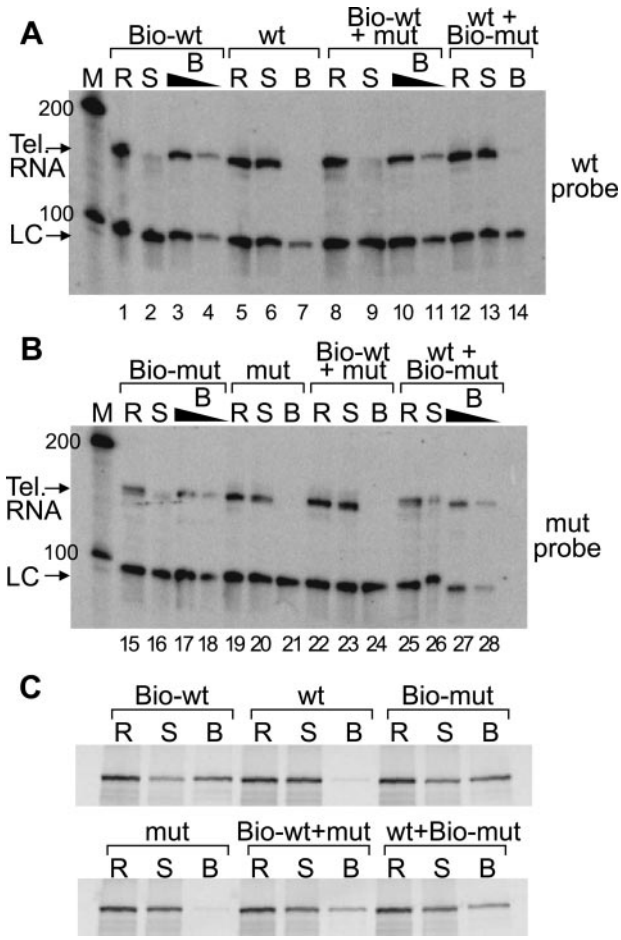

Figure 4. No pulldown of a marked telomerase RNA with biotinylated telomerase RNA. Wild-type or mutant (AC65–66UU) *Tetrahymena* telomerase RNA was in vitro transcribed and incubated with reticulocyte lysate-translated TERT. Bio-wt and Bio-mut indicate RNA biotinylated at the 5′ end, wild-type and mutant, respectively. The translation reactions were then either assayed separately or mixed and incubated for 15 min at 30°C, before pulldown on NeutrAvidin beads. (A) Northern blot of reticulocyte lysate translation reaction (R), supernatant (S), and beads (B) from the indicated reactions, using a probe specific for wild-type telomerase RNA. A 1:5 dilution of the beads was also loaded. Telomerase RNA and a 100-mer labeled DNA loading control (LC) are indicated with arrows. M, labeled RNA markers, with sizes in nucleotides. (B) Northern blot of the indicated samples, using a probe specific for mutant telomerase RNA. (C) The samples from A and B were run on an SDS-PAGE gel, and the ^{35}S-labeled TERT protein visualized with a PhosporImager.

Pulldown of Biotinylated DNA Primers Fails to Detect Telomerase Dimerization in Tetrahymena Extracts

To be able to assay for dimerization in native *Tetrahymena* extracts, we modified a procedure developed by Prescott and Blackburn (1997) for yeast telomerase, based on streptavidin purification of extended telomerase products. One biotinylated (Bio-Tet1) and one nonbiotinylated (Tet3) telomeric primer were extended separately or together in the presence of radiolabeled dGTP and ddTTP, and the reactions were subjected to pulldown with NeutrAvidin agarose beads. Tet3 is shorter than Bio-Tet1 so their extended products are distinguishable on an acrylamide gel (Figure 5A). As long as the telomerase complex stays bound to its product for long enough to complete the assay, the presence of a telomerase dimer will be revealed by pulldown of the nonbiotinylated products with the biotinylated ones.

When we carried out this assay using recombinant *Tetrahymena* telomerase, efficient pulldown of the biotinylated products was observed, there was little background binding of the nonbiotinylated products to the beads, and TERT protein remained bound to the beads (Figure 5, A and B). A significant amount of nonbiotinylated Tet3 products was recovered in the presence of Bio-Tet1 (marked with an asterisk in Figure 5A), which at first seemed to be evidence of dimerization. To control for the dependence on TERT of this effect, we carried out a similar reaction in which the primers were extended by telomerase and then the reaction was phenol/chloroform extracted before NeutrAvidin pulldown (Figure 5, C and D). Importantly, the same amount of Tet3 products was pulled down with Bio-Tet1 in the presence or absence of TERT. A similar result was obtained when the reactions were treated with both RNase and phenol/chloroform before NeutrAvidin pulldown (our unpublished data). This indicates that the observed binding is protein and RNA independent and is most likely mediated by intermolecular DNA interactions.

We speculated that the DNA interactions might involve the formation of intermolecular G-quartets, which is a well-known property of oligonucleotides containing runs of guanines in the presence of monovalent cations such as K^+ (Hardin *et al*., 2000). To test this, we repeated the experiment with nontelomeric primers consisting of repeats of the sequence GAGGTT, which should be much less likely to form G-quartets. Products of the nonbiotinylated nontelomeric primer were not recovered with the biotinylated products either in the presence or absence of protein (Figure 6, A and B, asterisks). Thus, we conclude that the interacting products seen in Figure 5 are an artifact most likely due to G-quartet formation and that no dimerization of native *Tetrahymena* telomerase is discernible using this assay.

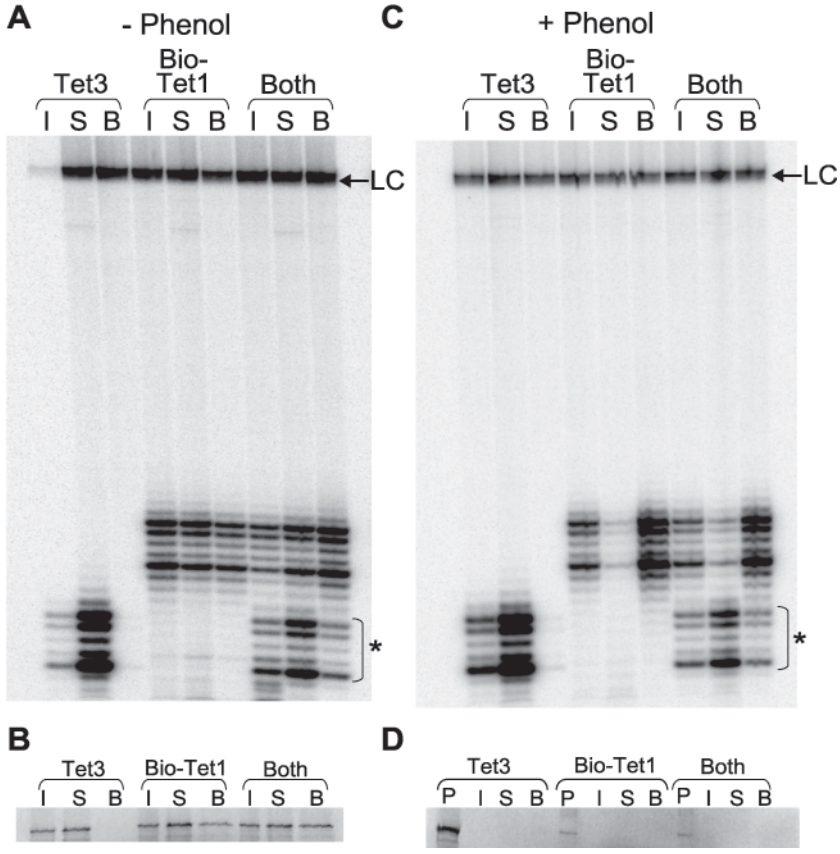

Figure 5. With recombinant telomerase, pulldown of a nonbiotinylated telomeric primer can be observed, but it is independent of TERT protein. Biotinylated telomeric DNA primer (Bio-Tet1) and nonbiotinylated primer (Tet3) were extended separately or together by recombinant *Tetrahymena* telomerase. The reactions were then subject to pulldown with NeutrAvidin beads directly (A and B) or extracted with phenol/chloroform before NeutrAvidin bead pulldown (C and D). Input (I), supernatant (S), and beads (B) from the pulldowns were electrophoresed on 10% denaturing polyacrylamide gels to visualize DNA products (A and C) and SDS-PAGE gels to visualize ^{35}S-labeled TERT protein (B and D). Lanes P in D are the protein samples before phenol/chloroform extraction. LC, 100-mer labeled DNA loading control.

The experiment in Figure 6 was carried out with extracts from mating *Tetrahymena*. Identical results were obtained with extracts from vegetatively growing *Tetrahymena* (our unpublished data). We verified that the telomerase complex was being pulled down with its reaction products by observing telomerase RNA on the beads by using a Northern blot (Figure 6C). Because *Tetrahymena* telomerase does not remain bound to its product as tightly as yeast telomerase (Greider, 1991; Prescott and Blackburn, 1997), only a small proportion of the telomerase RNA was recovered (5–20% in different experiments). Thus, this assay is less sensitive than it is with yeast telomerase; we estimate that we would be unable to detect dimers if they that constitute <40–50% of the active telomerase. We were unable to carry out this experiment using reticulocyte lysate telomerase because the amount of telomerase bound to product was even lower, possibly due to faster dissociation of recombinant telomerase from nontelomeric DNA. Attempts to verify that the RNA observed in Figure 6C remained in a complex with TERT were unsuccessful due to the lack of sensitivity of available *Tetrahymena* TERT antibodies, but at least at the beginning of the experiment native gel electrophoresis demonstrated that all of the telomerase RNA was in a protein complex (our unpublished data).

Free Tetrahymena Telomerase RNA Does Not Dimerize In Vitro

It has recently been demonstrated that under certain in vitro conditions, protein-free human telomerase RNA can form a dimer via an intermolecular pseudoknot (Ly *et al*., 2003). Because an intramolecular pseudoknot is conserved between ciliate and mammalian telomerase RNAs (Romero and Blackburn, 1991; Chen *et al*., 2000), we sought to determine whether *Tetrahymena* telomerase RNA can dimerize under similar conditions. When in vitro transcribed human telomerase RNA was prewarmed to 37°C for 2 h in the presence of 10 mM $MgCl_2$ before electrophoresis on a nondenaturing polyacrylamide gel, a slower migrating band was observed (Ly *et al*., 2003; Figure 7). This band is dependent on the prewarming treatment and has been shown to consist of two RNA molecules (Ly *et al*., 2003). When *Tetrahymena* telomerase RNA was subjected to the same conditions, no dimer was observed (Figure 7). A faint slower-migrating band was neither dependent on prewarming nor of a mobility consistent with an RNA dimer. Because recombinant *Tetrahymena* telomerase activity is maximal at 30°C and 1.25 mM $MgCl_2$ (Bryan, unpublished data), we also preincubated the RNA under these conditions, but again we did not observe a dimer (Figure 7).

DISCUSSION

The multimerization state of telomerase has implications for the mechanism of action of the enzyme. We therefore sought to determine whether *Tetrahymena* telomerase is active as a dimer or higher-level multimer, as has been shown for yeast, human, and *E. crassus* telomerase (Prescott and Blackburn, 1997; Armbruster *et al*., 2001; Beattie *et al*., 2001; Wenz *et al*., 2001; Wang *et al*., 2002). To our surprise, recombinant *Tetrahymena* telomerase eluted from a gel filtration column at a size consistent with it being a monomer. We were also unable to detect any dimerization of *Tetrahymena* telomerase

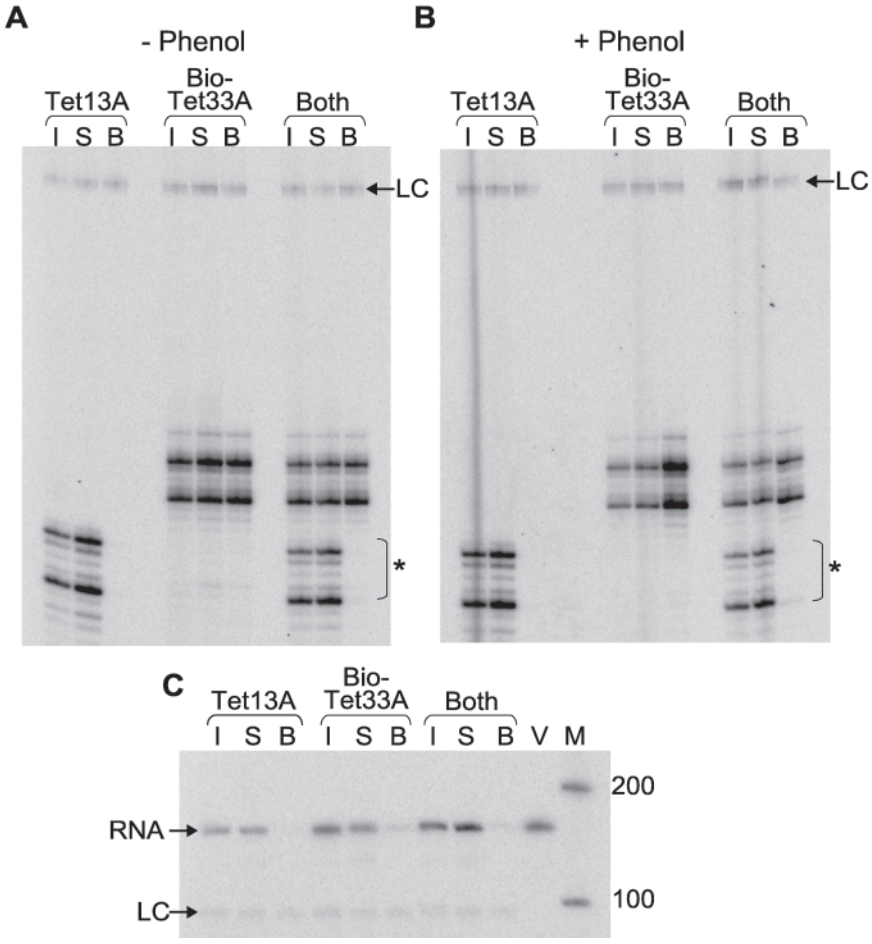

Figure 6. No pulldown of nonbiotinylated telomerase products with biotinylated ones using telomerase from *Tetrahymena* extracts. Biotinylated nontelomeric DNA primer (Bio-Tet33A) and nonbiotinylated primer (Tet13A) were extended separately or together by partially-purified native *Tetrahymena* telomerase. The reactions were then subject to pulldown with NeutrAvidin beads directly (A) or extracted with phenol/chloroform before NeutrAvidin bead pulldown (B). Input (I), supernatant (S), and beads (B) from the pulldowns were electrophoresed on 10% denaturing polyacrylamide gels to visualize DNA products. LC, 100-mer labeled DNA loading control. (C) Northern blot of the samples in A. Telomerase RNA and a 100-mer labeled DNA loading control (LC) are indicated with arrows. M, labeled RNA markers, with sizes in nucleotides. V, in vitro transcribed telomerase RNA (0.1 fmol).

by coprecipitation experiments, using tags on either the TERT protein or telomerase RNA. We therefore conclude that a majority, if not all, of the recombinant *Tetrahymena* telomerase in our reconstitution system is present as a monomeric complex of one TERT molecule with one telomerase RNA molecule, and that *Tetrahymena* telomerase does not need to dimerize to be active.

One intriguing rationale for the function of a telomerase dimer was that the extended DNA product could be passed from one active site to the other, allowing processive elongation (Wenz *et al*., 2001). Our reticulocyte-reconstituted *Tetrahymena* telomerase is, however, moderately processive (Bryan *et al*., 2000) even though it is monomeric. It remains possible that a dimer could be required to give higher processivity such as that observed with human telomerase or telomerase from *Tetrahymena* extracts.

Several potential artifactual explanations for our results seem unlikely. The amino-terminal tags present on the TERT proteins in our experiments might possibly alter the conformation of the protein and interfere with dimerization. However, amino-terminal tags on both copies of human TERT do not interfere with dimerization (Wenz *et al*., 2001). Similarly, it cannot be argued that our results are solely an artifact of the reticulocyte lysate system used for reconstitution, because dimerization has been observed for human telomerase expressed in the same way (Armbruster *et al*., 2001; Beattie *et al*., 2001; Moriarty *et al*., 2002).

One explanation for the results of our coimmunoprecipitation experiments (Figure 3) could be that active telomerase is in fact a dimer, but there is such a small proportion of telomerase in an active conformation in our reconstitution system that the dimerization is undetectable. The inefficiency of reconstitution of human telomerase in reticulocyte lysates has been given as an explanation for the failure to detect physical dimerization of human TERT by Western blotting in one study (Beattie *et al*., 2001). However, our gel filtration results indicate that reconstitution of *Tetrahymena* telomerase is quite efficient in this system. Approximately 30% of the TERT protein was in a complex with RNA in the experiment shown in Figure 1B, and this increased to ~60% when the amount of telomerase RNA was increased to the levels used in the experiment in Figure 3 (our unpublished results). If all of the TERT in Figure 3 were dimerized, the amount of signal in the lanes assaying dimerization (lanes 18 and 21) would be half as intense as those in the positive control lanes 15 and 23 (because lane 15, for example, would contain labeled FLAG-FLAG homodimers as well as labeled FLAG-T7 heterodimers, whereas any T7-T7 homodimers in lane 18 would be unlabeled and therefore undetectable). So even if only 60% of TERT was in an active conformation, the intensity of the band representing a dimer would be 30% of that of the controls (calculated as 0.5 × 60%), and we know that we can detect a band even fainter than 20% of the controls (e.g., lane 16). Therefore, we maintain that if all or

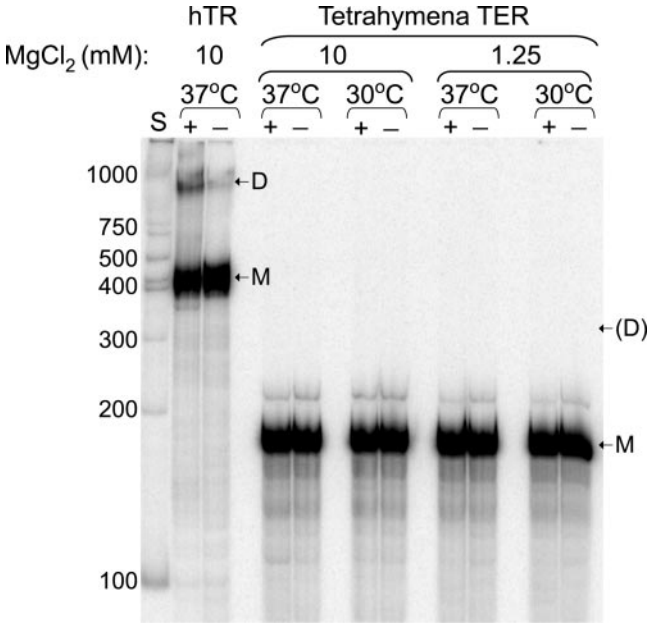

Figure 7. *Tetrahymena* telomerase RNA, unlike that of human, does not demonstrate a dimer on a native gel. In vitro transcribed telomerase RNAs from human (hTR) and *Tetrahymena* (TER) were end-labeled with ^{32}P, denatured at 95°C, adjusted to the indicated concentration of $MgCl_2$ and either heated to 37°C or 30°C (+) or kept on ice (−) for 2 h before electrophoresis in a 5% acrylamide native gel. S, labeled RNA markers, with sizes in nucleotides. M, monomer of each RNA species. D, dimer of hTR. (D), predicted migration of a dimer of *Tetrahymena* TER.

most of the active telomerase complex existed as a dimer, we would be able to see it in these experiments.

It remains possible that a majority of the active recombinant telomerase complex is monomeric and the remainder is dimeric, but is too small a proportion to be detected in our assays. This scenario nevertheless indicates that *Tetrahymena* telomerase does not need to form a stable dimer to be active.

We were also unable to detect dimerization of native telomerase from mating or vegetative *Tetrahymena* by using a primer pulldown assay. The sensitivity of this assay is lower with *Tetrahymena* telomerase than yeast, so it remains possible that the proportion of telomerase dimers is too low to be detectable. However, we remain confident that we would be able to detect if all or most telomerase existed as dimers, and our results again demonstrate that telomerase is active as a monomer. Furthermore, if human telomerase dimerization is mediated by an RNA-RNA interaction as has been suggested (Ly *et al*., 2003), *Tetrahymena* telomerase lacks the fundamental ability to form this dimer, at least under the conditions tested here.

Why would *Tetrahymena* telomerase differ from that of other species in what might be expected to be a fundamental property of the enzyme? One speculative explanation reflects a difference between telomere biology of ciliated protozoa and that of other organisms. It has been suggested that the role of telomerase dimerization might be to allow the coordinated extension of two telomere ends simultaneously, e.g., sister chromatids. During macronuclear development after mating in *Tetrahymena* and other ciliates, the macronuclear genome undergoes fragmentation into a large number of minichromosomes, which lack centromeres (reviewed in Prescott, 1994). A major role for telomerase in ciliates is to add telomeres to these fragments; levels of telomerase activity are higher during macronuclear development than during vegetative growth (Greider and Blackburn, 1985). This telomere addition probably does not require coordinated telomerase action on two chromosome ends, and hence ciliate telomerase may have had no evolutionary incentive to exist as anything other than a monomer. The recent proposal of telomerase dimerization in another ciliate, *E. crassus* (Wang *et al*., 2002), suggests that there may be heterogeneity even within the ciliates with respect to this property of telomerase.

We have shown that most recombinant and native *T. thermophila* telomerase complexes contain one molecule each of TERT and the RNA subunit. This demonstrates that this enzyme does not need to dimerize to be active or to accomplish processive extension of telomeric DNA.

ACKNOWLEDGMENTS

We thank Clare Fasching and Mike Jarstfer for comments on the manuscript, and Titia de Lange for suggesting the experiment in Figure 2. We thank Peter Bruns and Donna Cassidy-Hanley for *Tetrahymena* strains, Art Zaug and Jamie Sperger for several plasmids, Stefan Aigner for help with quantitating protein levels, Anne Gooding for T7 RNA polymerase and RNA oligonucleotide synthesis, Elaine Podell for DNA oligonucleotide synthesis, and YuMing Han for automated sequencing. This work was supported by the Howard Hughes Medical Institute, the Sylvia and Charles Viertel Charitable Foundation and the Wellcome Trust (Senior Research Fellowship GR066727MA).

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