Nucleic Acids Research, 1999, Vol. 27, No. 10 2227–2234

Tetrahymena telomerase ribonucleoprotein RNA–protein interactions

Chantal Autexier* and Ibtissem Triki

Bloomfield Centre for Research in Aging, Lady Davis Institute for Medical Research, The Sir Mortimer B. Davis-Jewish General Hospital, Montréal, Quebec H3T 1E2, Canada and Department of Anatomy and Cell Biology, McGill University, Montréal, Quebec H3A 2B2, Canada

Received October 16, 1998; Revised and Accepted March 15, 1999

ABSTRACT

Telomerase is an enzyme that is essential for the replication and maintenance of chromosomal termini. It is a ribonucleoprotein consisting of a catalytic subunit, one or more associated proteins, and an integral RNA subunit that serves as a template for the synthesis of telomeric repeats. We identified a *Tetrahymena* telomerase RNA–protein complex by an electrophoretic mobility shift assay, using telomerase partially purified from whole cell extracts and radiolabeled, *in vitro* transcribed wild-type *Tetrahymena* telomerase RNA. Complex formation was specific as unlabeled *Tetrahymena* telomerase RNA, but not *Escherichia coli* ribosomal RNAs, competitively inhibited complex formation. Binding required concentrations of $MgCl_2$ of at least 10 mM and occurred over a wide range of potassium glutamate concentrations (20–220 mM). The RNA–protein complex was optimally reconstituted with a 30°C preincubation for ≤5 min, prior to electrophoresis. Certain *Tetrahymena* telomerase RNAs containing deletions of structures and sequences previously predicted to be involved in protein binding were unable to competitively and specifically inhibit complex formation, suggesting a role in protein binding for the deleted residues or structures.

INTRODUCTION

Telomerase is an enzyme that is essential for the replication and maintenance of chromosomal termini (1). It is a ribonucleoprotein consisting of a catalytic subunit, one or more associated proteins, and an integral RNA subunit that serves as a template for the synthesis of telomeric repeats (2). In *Tetrahymena*, the telomerase RNA is 159 nt long and assumes a secondary structure that includes four conserved helices and a pseudoknot structure (3,4) (Fig. 1). The template domain contains the sequence 5′-CAACCCCAA-3′ that directs the synthesis of specific telomeric repeats (TTGGGG)n onto DNA (5,6).

The methylation patterns, from chemical modification experiments, of protein-free and protein-bound telomerase RNA have provided insights into the segments of RNA that may contact protein (7,8). The hairpin loop of stem III (residues 89 and 90) which is adjacent to the pseudoknot, the GA bulge in stem IV (residues 121 and 122) and three sites around residues 15 and 16, 39 and 62 are implicated in protein binding (Fig. 1). Telomerase reconstituted *in vitro* with RNAs containing mutations in some of these residues has reduced activity relative to telomerase reconstituted with wild-type telomerase RNA. These residues may play a role in telomerase function, perhaps indirectly, by binding to telomerase protein components (9).

Two *Tetrahymena* telomerase proteins were identified on the basis of copurification and coimmunoprecipitation with both telomerase activity and the *Tetrahymena* telomerase RNA (10). p80, which has homologs in human, mouse and rat, crosslinks to *Tetrahymena* telomerase RNA (10–12). p95 was shown to crosslink specifically to telomeric DNA primers in an RNA-independent manner (10). Studies with recombinant p80 and p95 indicate that each protein interacts directly with the telomerase RNA, that the two proteins form a complex independently of RNA and that the RNA-binding affinity of the p80/p95 complex is greater than either of the individual proteins (13).

The *in vivo* interaction of telomerase proteins with telomerase RNA is not clearly understood. The *in vitro* interaction of *Tetrahymena* p80/p95 with telomerase RNA shows a limited degree of specificity (13). In *Euplotes*, yeast, human and mouse, catalytic reverse transcriptase components of telomerase, known as p123, Est2p, hTERT and mTERT, respectively, have recently been identified (14–21). At the time our studies were undertaken, no telomerase reverse transcriptase (TERT) homolog had yet been reported for *Tetrahymena*. Recently, such a homolog, p133, was identified (22,23). Human and *Tetrahymena* telomerase activity can be reconstituted, *in vitro*, by the expression of only the catalytic subunit of telomerase and the telomerase RNA in a rabbit reticulocyte lysate. These data suggest that direct protein–RNA interactions occur between the telomerase RNA and p133/hTERT (22,24,25).

To investigate the interaction of *Tetrahymena* telomerase proteins and associated proteins, including p80, p95, a TERT homolog and any other, as yet unidentified proteins with the *Tetrahymena* telomerase RNA, we developed an electrophoretic mobility shift assay. A specific RNA–protein complex was identified and characterized. Certain *Tetrahymena* telomerase

*To whom correspondence should be addressed at: Lady Davis Institute for Medical Research, The Sir Mortimer B. Davis-Jewish General Hospital, 3755 Côte Ste. Catherine Road, Montréal, Quebec H3T 1E2, Canada. Tel: +1 514 340 8260; Fax: +1 514 340 8295; Email: mcfj@musica.mcgill.ca

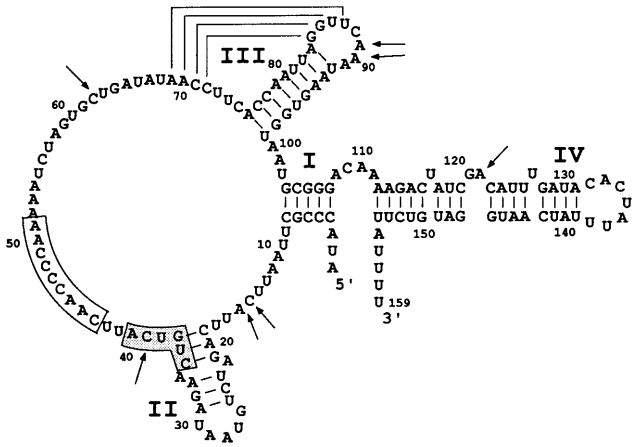

Figure 1. Secondary structure of the *Tetrahymena* telomerase RNA, including the pseudoknot structure (brackets) (3,4). The template and alignment regions (open box) 5′-CAACCCCAA-3′ span residues 43–51. The upstream conserved region 5′-(CU)GUCA-3′ (residues 35–40, shaded box), regulates the 5′ boundary of the template (27,38). Arrows highlight residues that have been implicated in protein binding (38).

RNAs containing deletions within structures and sequences previously predicted to be involved in protein binding did not competitively inhibit complex formation. These results suggest a role in protein binding for the deleted residues or structures.

MATERIALS AND METHODS

Preparation of *Tetrahymena* telomerase

Tetrahymena telomerase was purified using a protocol modified from Collins *et al*. (10) and described in Autexier and Greider (9). Briefly, the S130 extract prepared from 72 l of 4.0×10^5 cells/ml harvested *Tetrahymena* cells was loaded onto a ceramic hydroxypatite (American International Chemical) column equilibrated in T2MG (20 mM Tris–HCl pH 8.0, 1 mM $MgCl_2$, 10% glycerol) and eluted with a linear salt gradient (0–0.2 M K_2HPO_4) in T2MG. Fractions were assayed for telomerase activity as previously described (9). Active fractions were loaded, after diluting 4-fold in T2MG, onto a spermine agarose (Sigma) column equilibrated in T2MG with 0.15 M potassium glutamate (Kglu) and eluted with 0.65 M Kglu in T2MG. The active fractions were pooled, the Kglu concentration adjusted to 0.6 M, and then loaded onto a phenyl-Sepharose (Pharmacia) column equilibrated in T2MG with 0.6 M Kglu. Proteins were eluted with 1% Triton X-100 in T2MG. Finally, to concentrate the telomerase activity, fractions containing maximal telomerase activity were pooled and loaded onto a DEAE-agarose (Bio-Rad) column. Proteins were eluted in T2MG with 0.4 M Kglu. The peak of telomerase activity (1.5 ml) contained 0.45 mg of protein. DEAE-agarose-purified telomerase (0.3 mg/ml) was diluted 20-fold (Figs 2B–4) or a different DEAE-agarose-purified telomerase (0.6 mg/ml) was diluted 5-fold (Fig. 2A, lanes 7–8 and Figs 5–7) with T2MG before use in the RNA band shift assays. The preparations remained active for at least 1 year when stored at –70°C. Protein concentrations were determined by a Bradford assay with Bio-Rad dye reagent.

Preparation of RNAs

Plasmid pT7159, containing the gene encoding the *Tetrahymena thermophila* telomerase RNA was digested using *Fok*I (26). Plasmids containing mutant versions of the *Tetrahymena* telomerase RNA gene were digested with *Eco*RI (pt146), *Xba*I and *Bam*HI (pΔ5′36, pt75) and *Fok*I (p38–40AGT) as previously described (9,27). Plasmid phTR+1, containing the gene encoding the human telomerase RNA was digested using *Fsp*I (28). Plasmid pT7ETR, a kind gift of Dorothy Shippen, and containing the gene encoding the *Euplotes crassus* telomerase RNA, was linearized with *Bst*BI. For the preparation of unlabeled RNA, standard *in vitro* transcription reaction conditions recommended by the T7 RNA polymerase manufacturer were used (Stratagene or New England BioLabs). The transcription reactions were treated with 3 U RNase-free DNase (Pharmacia)/μg DNA for 10 min. The RNA concentrations were measured by fluorometry or spectrophotometry. The integrity and size of the RNAs were determined by staining with ethidium bromide. t75 and t146 RNA contain the first 5′ 75 and 146 residues of the *Tetrahymena* telomerase RNA, respectively (9). Δ5′36 RNA contains a deletion of 36 residues at the 5′ end of the *Tetrahymena* telomerase RNA. 38–40AGU RNA substitutes residues U, C and A at positions 38–40 in the *Tetrahymena* telomerase RNA, with residues A, G and U (9,27). Labeled RNA was prepared using two different methods. Unlabeled *in vitro Tetrahymena* telomerase RNA was dephosphorylated by treating 1.4 μg (25 pmol of 5′ ends) with 25 U of calf intestinal alkaline phosphatase (CIAP-New England BioLabs-NEB) in 1× NEB buffer 3 (50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, pH 7.9) for 60 min at 50°C. The reaction was heat inactivated at 75°C for 10 min in 5 mM EDTA pH 8.0, phenol extracted and ethanol precipitated using 0.3 M sodium acetate pH 5.2 (NaOAc). The RNA was resuspended at a concentration of 0.4 μg/μl in diethyl pyrocarbonate (DEPC)-treated H_2O. 400 ng of dephosphorylated RNA was radiolabeled with 10 μl (100 μCi) [γ-^{32}P]ATP (6000 Ci/mmol; NEN) and 10 U polynucleotide kinase (PNK-Pharmacia) as recommended by the manufacturer for 20 min at 37°C. The RNA was phenol extracted and ethanol precipitated and the labeled telomerase RNA was resuspended at 16 ng/μl in DEPC-treated H_2O. Typically 1–2 μl of a 1:10 dilution was used per RNA band shift reaction. Generally, unincorporated radiolabeled nucleotides were first removed by passing the RNA through Sephadex G-25 medium resin (NAP-10 column from Pharmacia) or through an anion exchange resin (Quiagen RNA/DNA kit), and gel purification. Briefly, the RNA was resuspended in formamide, heated to 75°C for 5 min prior to loading on a 6% polyacrylamide 7 M urea 0.6× TBE gel (280 V, 13 mA for 2 h). The band was excised and the RNA was eluted from the gel by crushing and soaking in 0.75 M ammonium acetate, 1 mM EDTA, 40 U RNasin overnight at 37°C, or 1 h at 65°C followed by phenol–chloroform extraction and ethanol precipitation. The RNA was washed three times with 70% ethanol prior to resuspending in DEPC-treated H_2O. Alternatively, the RNA was radiolabeled during the transcription of 1 μg of *Fok*I digested pT7159 plasmid with T7 RNA polymerase (25 U) in a reaction containing 40 mM Tris–HCl pH 8.0, 8 mM $MgCl_2$, 50 mM NaCl, 2 mM spermidine, 30 mM DTT, 0.1 μg/μl BSA, 1 U/μl RNasin (Promega), 500 μM each ATP, GTP, CTP, 10 μM UTP and 100 μCi [α-^{32}P]UTP (800 Ci/mmol; NEN). After a 1 h incubation at 37°C, the RNA was treated with RNase-free DNase I (Pharmacia; 7.5 U/μg of

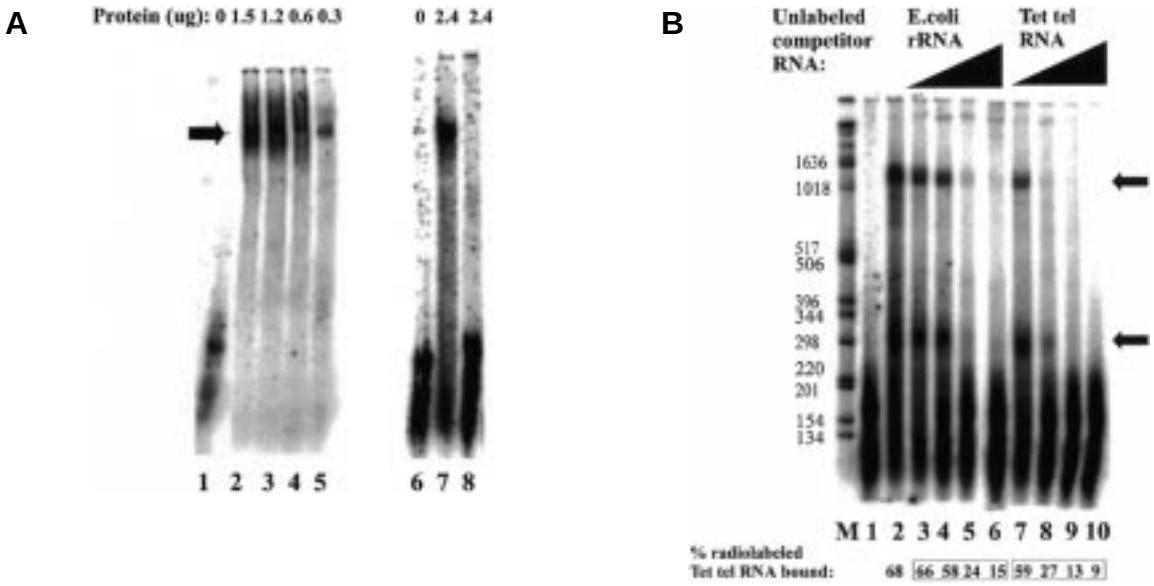

Figure 2. RNA band shift assay with *Tetrahymena* telomerase. (**A**) 1.6 ng of ^{32}P-labeled *Tetrahymena* telomerase RNA was incubated with the following concentrations of telomerase as follows: lanes 1 and 6, 0 µg; lane 2, 1.5 µg; lane 3, 1.2 µg; lane 4, 0.6 µg; and lane 5, 0.3 µg. The concentration of $MgCl_2$ in this standard assay was 5 mM. In lanes 7 and 8, 2.4 µg of telomerase was incubated with ^{32}P-labeled RNA (lane 7) or pre-treated with proteinase K prior to the addition of RNasin and ^{32}P-labeled RNA (lane 8). The concentration of $MgCl_2$ in lanes 7 and 8 was 10 mM. RNA–protein complexes were separated from free RNA on a 4% composite acrylamide/agarose gel, dried and exposed to a PhosphorImager screen overnight as described in Materials and Methods. (**B**) 1.6 ng of ^{32}P-labeled *Tetrahymena* telomerase RNA incubated with 0 µg (lane 1), 0.3 µg of telomerase (lanes 2–10) and increasing amounts (1, 10 and 100 ng, and 1 µg) of the unlabeled specific *Tetrahymena* telomerase RNA (Tet tel RNA, lanes 7–10) or non-specific *E.coli* 16S and 23S rRNA (*E.coli* rRNA, lanes 3–6). The unlabeled RNAs were added prior to the radiolabeled RNA. The concentration of $MgCl_2$ in this standard assay was 5 mM. M represents 5′-end labeled 1 kb molecular weight DNA standard (bp). The RNA–protein complexes are highlighted with arrows. The percentage of radiolabeled RNA bound (both complexes) as a fraction of the total radiolabeled RNA in each reaction is indicated under the respective lanes.

DNA) and gel purified. *Escherichia coli* 16S and 23S rRNA were purchased from Sigma, *E.coli* 5S rRNA from Boehringer Mannheim and the yeast tRNA from Sigma.

RNA band shift assay

For the standard RNA band shift assay, DEAE-agarose-purified *Tetrahymena* telomerase was diluted 20-fold in T2MG and 20 µl was treated with 5 mM EDTA and incubated for 5 min at 30°C with ^{32}P-labeled *Tetrahymena* telomerase RNA (1.6 ng). In competition experiments, unlabeled RNA was added prior (Fig. 2B), or simultaneously (Figs 5–7) with the labeled wild-type *Tetrahymena* telomerase RNA. Reactions were placed on ice and 5 or 10 mM $MgCl_2$ was added. Loading buffer (4 µl; 0.3–0.5× TBE, 50% glycerol) was added to each sample. Pre-treatment of the EDTA-treated extract with proteinase K (0.8 µg/µl) (Sigma) was performed for 10 min at 30°C, prior to the addition of RNasin (Promega) and radiolabeled RNA. A radiolabeled molecular weight DNA standard (1 kb, Gibco BRL) was loaded in the same loading buffer containing xylene cyanol and bromophenol blue. Reactions were electrophoresed through a composite gel system modified from Nelson and Green (29). The gel was 4% acrylamide, 0.1% piperazine di-acrylamide (Bio-Rad), 0.5% agarose, 10% glycerol, 0.3–0.5× TBE (0.8 mm thick; size: 17 × 17 cm). The running buffer was 0.3 or 0.5× TBE (0.5× TBE: 45 mM Tris–borate, 1 mM EDTA). The gel was pre-run for 30–60 min at 60 V and run at 12 mA (180–250 V) for ~5 h at 4°C, until the bromophenol blue was out of the gel. Gels were dried and exposed to film at –20 or –70°C overnight, or on PhosphorImager plates (Molecular Dynamics). On occasion, films were scanned using a Molecular Dynamics Densitometer. The electrophoretic mobility shift assays were analyzed with a PhosphorImager to quantitate the fraction of bound RNA. The amount of competitor RNA resulting in a percentage inhibition of binding was calculated by normalization to the standard binding reaction (without competitor). A non-linear curve fit using the Hill model [%Inhib = $(I_{max} \times [I]^n)/(IC_{50}^n + [I]^n)$] was then applied to the percentage inhibition-concentration data and 50% effective concentration (IC_{50}) was calculated by the sum of least squares using Microsoft Excel Solver (Microsoft Corp., Seattle, WA). IC_{50} values were calculated from a number of experiments (two to three) and are expressed with the observed standard deviation (±SD).

RESULTS

Identification and characterization of a specific *Tetrahymena* telomerase RNA–protein complex

To investigate the interaction of *Tetrahymena* telomerase and associated proteins, including p80, p95, a TERT homolog and any other, as yet unidentified proteins, with the *Tetrahymena* telomerase RNA, we developed an electrophoretic mobility shift assay. We used active telomerase partially purified from whole cell extracts and radiolabeled, *in vitro* transcribed wild-type *Tetrahymena* telomerase RNA. The integrity of many ribonucleoproteins (RNPs) is dependent on divalent cations. Chelating agents such as EDTA have been used to partially unfold a number of RNPs

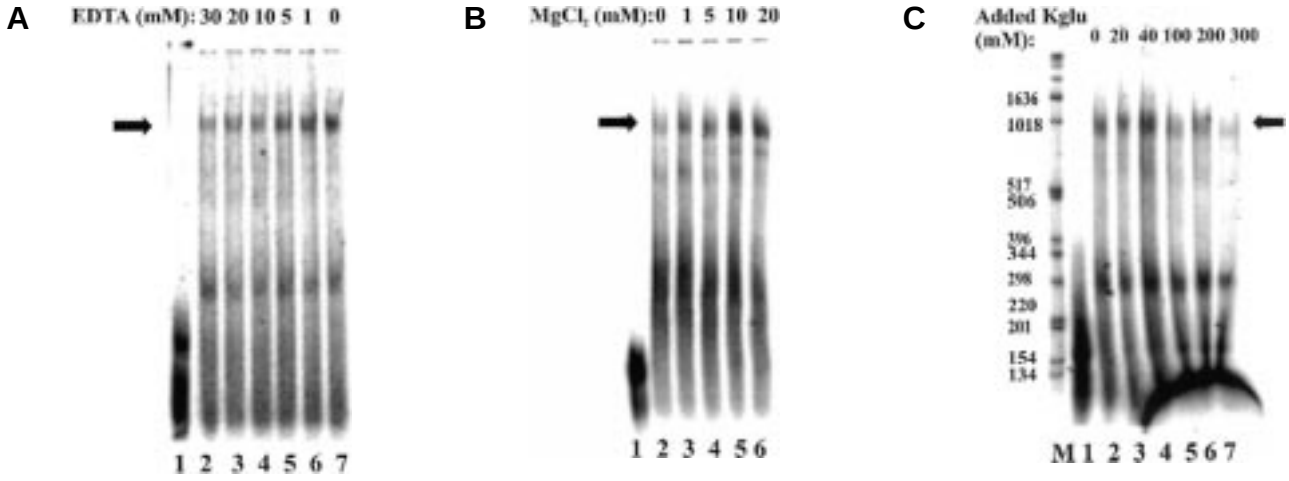

Figure 3. Formation of the *Tetrahymena* telomerase RNA–protein complex. (**A**) Titration of EDTA concentration. A standard RNA band shift assay (see legend to Fig. 2 and Materials and Methods) was performed (5 mM $MgCl_2$) and the concentration of EDTA was varied as follows: lane 2, 30 mM; lane 3, 20 mM; lane 4, 10 mM; lane 5, 5 mM; lane 6, 1 mM; lane 7, no EDTA. Lane 1: labeled telomerase RNA not incubated with extract. (**B**) $MgCl_2$ titration. A standard RNA band shift assay was performed with the following concentrations of $MgCl_2$: lane 2, no $MgCl_2$; lane 3, 1 mM; lane 4, 5 mM; lane 5, 10 mM; lane 6, 20 mM. Lane 1: labeled telomerase RNA not incubated with extract. (**C**) Salt (potassium glutamate, Kglu) titration. A standard RNA band shift assay was performed (5 mM $MgCl_2$) and the amount of added Kglu was varied as follows: lane 2, no added Kglu; lane 3, 20 mM; lane 4, 40 mM; lane 5, 100 mM; lane 6, 200 mM; and lane 7, 300 mM. M represents 5′-end labeled 1 kb molecular weight DNA standard (bp). Lane 1 represents labeled telomerase RNA not incubated with extract. The RNA–protein complexes are highlighted by arrows.

including ribosomal subunits, RNase P, small cytoplasmic RNPs and SRP (30–35). Telomerase extract purified by ceramic hydroxyapatite, spermine-agarose, phenyl-Sepharose and DEAE-agarose chromotography was incubated with 1.6 ng of ^{32}P-labeled *Tetrahymena* telomerase RNA and 5 mM EDTA. Following a 5 min incubation at 30°C, 5 mM $MgCl_2$ was added prior to electrophoresis on a native composite agarose/acrylamide gel. An RNA-dependent complex was identified that migrated slightly slower than a 1 kb DNA marker (Fig. 2). The complex migrated as a sharp band with as little as 300 ng of total protein extract (Fig. 2A, lane 5). Pre-treatment of the extract with proteinase K abolished complex formation indicating that protein component(s) are required for complex formation (Fig. 2A, lanes 7 and 8). On occasion, an RNA–protein complex of faster mobility that co-migrated with a 300 bp marker was also detected (Fig. 2B). Free *Tetrahymena* telomerase RNA generally migrated as two forms, at ~159 and 120 nt, presumably due the formation of different secondary structures. Gel purification of the RNA, and/or heating the RNA and cooling it slowly to room temperature did not reduce the formation of the faster migrating RNA.

The *Tetrahymena* telomerase RNA band shift assay was repeated with excess unlabeled specific *Tetrahymena* telomerase RNA or non-specific RNA, to determine whether binding was specific. The formation of bound complexes was reduced in the presence of unlabeled specific RNA. A 1-fold and 10-fold excess of specific RNA reduced binding 41 and 73%, respectively (Fig. 2B, compare lanes 7 and 8 with lane 2). A 10-fold excess of *E.coli* 16S and 23S rRNA only decreased complex formation by 42% (Fig. 2B, lane 4). A specific complex was still detected in the presence of 100-fold and 1000-fold excess of unlabeled *E.coli* rRNA (24 and 15% of control RNA bound, respectively; Fig. 2B, lanes 5 and 6). These competition experiments (Fig. 2B) were performed under stringent conditions that required the addition of unlabeled competitor RNA to the telomerase prior to the specific labeled RNA. The experiments were also performed with the simultaneous addition of the competitor and radiolabeled RNA to the telomerase. Though the amount of competitor RNAs required to reduce complex formation was higher when added simultaneously, the relative specificity of the two competitor RNAs was similar, regardless of this order of addition (data not shown). The amount of competitor RNA resulting in a 50% inhibition of binding was 24.8 and 140.3 ng for wild-type *Tetrahymena* telomerase and *E.coli* rRNA, respectively. To compare RNAs of similar size, the experiment was repeated with *E.coli* 5S rRNA (120 nt) and yeast tRNA (79 nt) as competitor RNAs, and similar results were obtained (data not shown).

To characterize the ionic requirements for the formation of the specific *Tetrahymena* telomerase RNA–protein complex, titrations of EDTA, $MgCl_2$ and potassium glutamate were performed. Complex formation was optimal with <5 mM EDTA pre-treatment ($MgCl_2$ was kept constant at 5 mM). Excessive EDTA (30 mM) pre-treatment inhibits complex formation by 11% compared to no EDTA (Fig. 3A). In the presence of 5 mM EDTA, complex reconstitution optimally required 10–20 mM $MgCl_2$ (Fig. 3B). Complex formation was inhibited by 18% in the absence of $MgCl_2$. The DEAE-agarose-purified telomerase used in the RNA band shift assays contains 20 mM Kglu, after a 20-fold dilution in T2MG. The addition of 300 mM Kglu inhibits complex formation by 14% compared to 40 mM Kglu. A final Kglu concentration of 20–60 mM was optimal for complex formation (Fig. 3C, lanes 2–4).

To determine the optimal temperature and time of incubation for the formation of the specific *Tetrahymena* telomerase RNA–protein complex, the standard RNA gel shift assay was performed at different temperatures and times of incubation (Fig. 4). Incubation at 4°C (on ice) was tested at varying times, and complex formation was maximal at 15 min (49% RNA bound

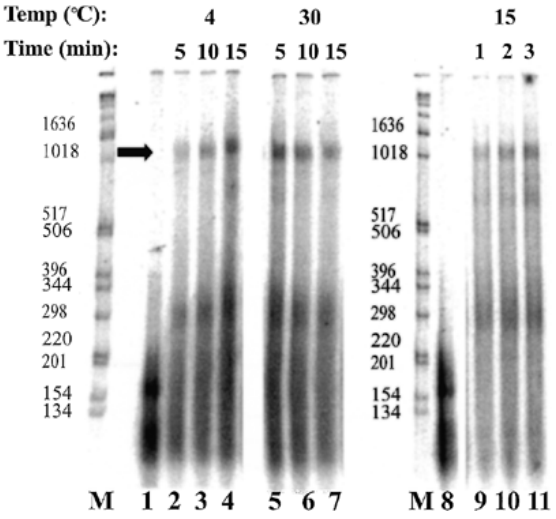

Figure 4. The effect of temperature on *Tetrahymena* telomerase RNA–protein complex formation. Standard band shift assays were performed (5 mM $MgCl_2$) but the time and temperature of incubation were varied as follows: lanes 2–4, 4°C (on ice) for 5, 10 and 15 min; lanes 5–7, 30°C for 5, 10 and 15 min; lanes 9–11, 15°C for 1, 2 and 3 min. In lanes 9–11, 40 mM Kglu was also added in the standard assay. Lanes 1 and 8 represents labeled telomerase RNA not incubated with extract. M represents 5′-end labeled 1 kb molecular weight DNA standard. The RNA–protein complex is indicated by the arrow.

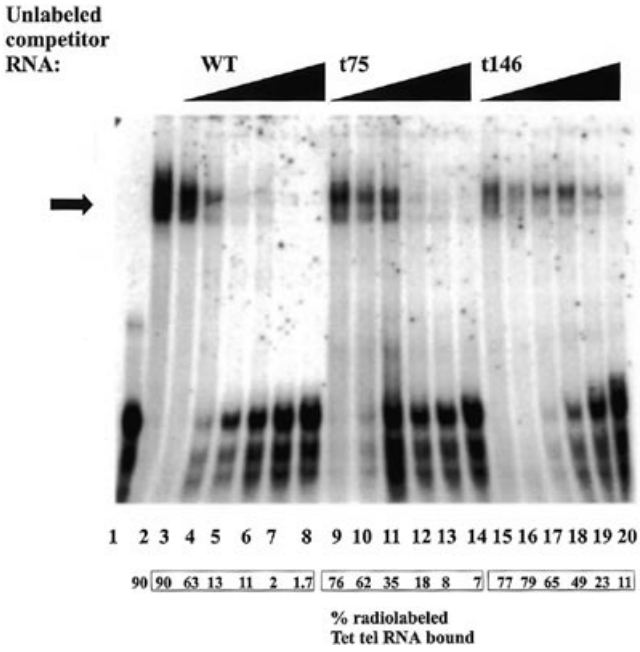

Figure 5. Non-specific inhibition of *Tetrahymena* telomerase RNP formation. Standard RNA band shift assays were performed (2.4 µg protein; 10 mM $MgCl_2$) with increasing amounts of unlabeled RNA (1, 10, 50, 100, 200 and 500 ng). Lanes 3–8, wild-type *Tetrahymena* telomerase RNA; lanes 9–14, t75 *Tetrahymena* telomerase RNA; lanes 15–20, t146 *Tetrahymena* telomerase RNA. The unlabeled and radiolabeled RNAs were added simultaneously to the extract. Lane 1 represents labeled telomerase RNA not incubated with extract. Lane 2 represents labeled telomerase RNA incubated with extract but without competitor RNA. The RNA–protein complex is indicated by the arrow. The percentage of the total radiolabeled *Tetrahymena* telomerase RNA bound is indicated for each lane.

compared to 39% at 5 min) (lanes 2–4). Incubation at 15°C was tested for 1, 2 and 3 min, and complex formation was maximal at 3 min (52% RNA bound compared to 42% at 1 min) (lanes 9–11). Incubation at 30°C was tested for 5, 10 and 15 min and complex formation was suboptimal beyond 5 min (52% RNA bound compared to 48% at 15 min) (lanes 5–7). Incubation at 30°C for 30 s, 1, 2 and 5 min revealed that complex formation was optimal in the first minute and that binding was reduced with longer incubations (data not shown).

Protein-binding domains of the *Tetrahymena* telomerase RNA

In order to identify protein-binding domains of the *Tetrahymena* telomerase RNA, competition experiments were performed using the standard RNA band shift assay and telomerase RNA mutants. A series of terminal deletions and substitutions in the *Tetrahymena* telomerase RNA have previously been tested for their ability to reconstitute telomerase activity in an *in vitro* assay (9). Several mutants did not reconstitute, or reconstituted reduced levels of activity relative to telomerase reconstituted with wild-type RNA. Many of the telomerase RNA mutations were in structures predicted to be involved in protein binding (7,8). To determine whether defects in reconstituting telomerase activity were due to defects in protein binding, and to identify protein-binding domains in the telomerase RNA, several of these mutants were tested for their ability to competitively inhibit the formation of the *Tetrahymena* telomerase RNA–protein complex.

Tetrahymena telomerase RNA–protein complex formation is non-specifically inhibited by mutant *Tetrahymena* telomerase RNAs containing deletions of 13 and 84 residues at the 3′ end of the RNA (t146 and t75, respectively) (Fig. 5). In separate experiments, unlabeled competitor t75 or t146 RNAs were added to the protein fraction prior to or simultaneously with the radiolabeled wild-type *Tetrahymena* telomerase RNA. The amount of competitor RNA required to inhibit complex formation was slightly higher when the competitor and radiolabeled RNA were added simultaneously. However, the relative specificity of the three competitor RNAs was similar, regardless of the order of addition of the competitor RNA and radiolabeled RNA. The amount of competitor RNA resulting in a 50% inhibition (±SD) of binding was 18.1 (±9.7), 37.8 (±19.9) and 87.8 (±8.1) ng for wild-type, t75 and t146 RNAs, respectively. Competitive inhibition was most efficient with the simultaneous addition of wild-type *Tetrahymena* telomerase RNA. A 10- and 50-fold excess of unlabeled RNA reduced complex formation by 37 and 87%, respectively (lanes 4 and 5). The deletion of both stem loops III and IV (t75 RNA) required a 200-fold excess of this mutant RNA to inhibit complex formation by 92% (Fig. 5, lane 13). Complex formation was inhibited by 89% with a 500-fold excess of t146 RNA (lane 20). Both of these mutant RNAs were compromised to different extents in their ability to compete for the binding of wild-type telomerase RNA to protein in the telomerase extract. These results suggest a role in protein binding for the deleted residues or structures.

Tetrahymena telomerase RNA mutants with a 5′-terminal deletion of 36 residues (Δ5′36) and substitutions at positions 38–40 (38–40AGU) competitively inhibited the formation of the telomerase RNA–protein complex to the same extent as unmodified wild-type RNA (Fig. 6). A 50-fold excess of wild-type *Tetrahymena*

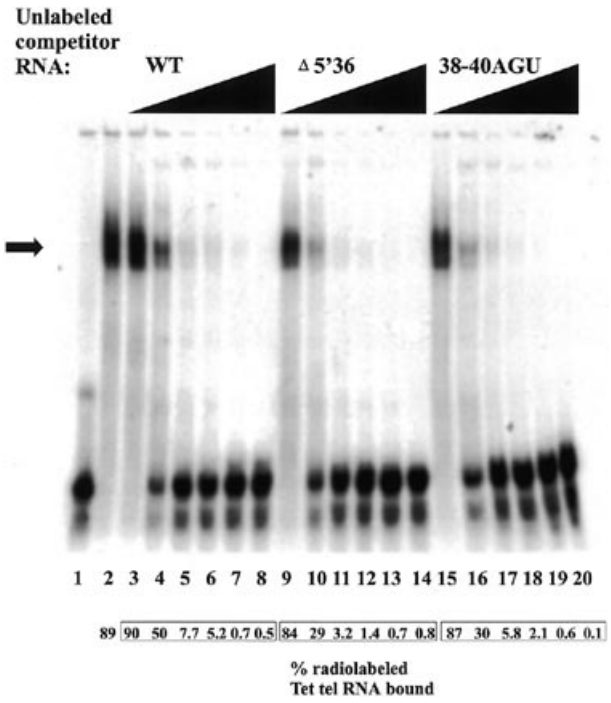

Figure 6. Specific inhibition of *Tetrahymena* telomerase RNP formation. Standard RNA band shift assays were performed (2.4 µg protein; 10 mM $MgCl_2$) with increasing amounts of the indicated unlabeled RNA (1, 10, 50, 100, 200 and 500 ng) added to the reactions as follows: wild-type RNA (lanes 3–8); a mutant containing a deletion at the 5′ end (Δ5′36) of the RNA (lanes 9–14); and a mutant telomerase RNA (38–40AGU) containing a substitution in the upstream conserved region (lanes 15–20). The unlabeled RNA and radiolabeled RNA were added simultaneously to the extract. Lane 1, labeled telomerase RNA not incubated with extract; lane 2, labeled telomerase RNA incubated with extract but without competitor RNA. The RNA–protein complex is indicated by the arrow. The percentage of the total radiolabeled RNA bound is indicated for each lane.

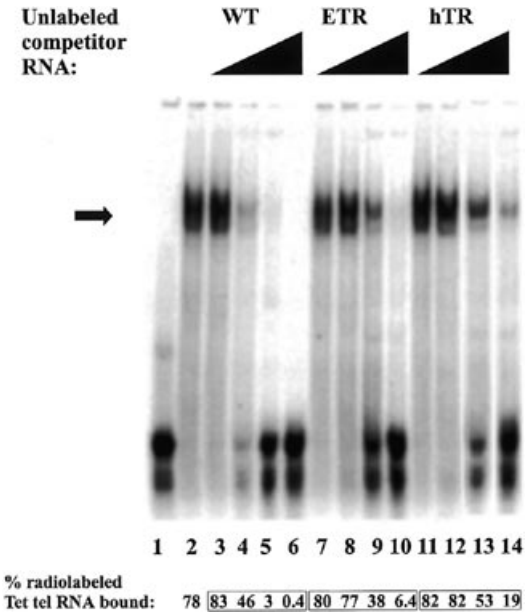

Figure 7. Inhibition of the *Tetrahymena* telomerase RNA–protein complex by *Euplotes* and human telomerase RNAs. Standard RNA band shift assays were performed (2.4 µg protein; 10 mM $MgCl_2$), with increasing amounts of unlabeled RNA (1, 10, and 100 ng, and 1 µg) added to the reactions as follows: lanes 3–6, wild-type *Tetrahymena* telomerase RNA; lanes 7–10, *E.crassus* telomerase; lanes 11–14, human telomerase RNA. The unlabeled RNAs were added to the extract simultaneously with the radiolabeled RNA. Lane 1, labeled telomerase RNA not incubated with extract; lane 2, labeled telomerase RNA incubated with extract but without competitor RNA. The RNA–protein complex is indicated by the arrow. The percentage of the total radiolabeled RNA bound RNA is indicated for each lane.

telomerase RNA significantly inhibited complex formation by 92.3% (lane 5). Inhibition (96.8%) of complex formation by RNA that is deleted in both stem I and stem loop II (Δ5′36) also occurred with a 50-fold excess of mutant RNA (lane 11). The inhibition (94.2%) of complex formation by the 38–40AGU RNA was as efficient as wild-type telomerase RNA, requiring a 50-fold excess of mutant RNA (lane 17). The amount of competitor RNA resulting in a 50% inhibition (±SD) of binding was 11.5 (±0.2), 4.7 (±1.3) and 6.7 ng for wild-type, Δ5′36 and 38–40AGU RNAs, respectively.

Competitive inhibition of the *Tetrahymena* telomerase RNA–protein complex by the *Euplotes* telomerase RNA

In a test for species specificity of the *Tetrahymena* telomerase RNA–protein interaction, RNA binding assays were performed in the presence of either *E.crassus* or human telomerase RNAs. The *E.crassus* and human telomerase RNAs are 191 and 445 nt in length, respectively (36,37). These RNAs have little primary sequence homology, but the telomerase RNAs of *Euplotes* and *Tetrahymena* fold into similar secondary structures (36,38). Both the *Euplotes* and human telomerase RNAs inhibited complex formation, although not as efficiently as the *Tetrahymena* telomerase RNA (Fig. 7, compare lane 4 to lanes 8 and 12 at 10-fold excess unlabeled RNA). Significant inhibition of complex formation by the *Euplotes* telomerase RNA occurred in the 10–100-fold range of excess of mutant RNA (23–62% inhibition in this range, lanes 8 and 9), whereas inhibition by human telomerase RNA was less efficient (18–47% inhibition, lanes 12 and 13) in the identical range. The order of addition also had no effect on this competition as similar results were obtained when the ETR and hTR RNAs were added prior to the radiolabeled *Tetrahymena* telomerase RNA (data not shown). The amount of competitor RNA resulting in a 50% inhibition (±SD) of binding was 10.3 (±0.1), 81.6 (±11.4) and 101.9 (±6.2) ng for wild-type, ETR and hTR RNAs, respectively.

DISCUSSION

We have identified and characterized a specific *Tetrahymena* telomerase RNA–protein complex. Active telomerase partially purified over several chromatographic resins is likely to contain all the telomerase proteins and any associated proteins that are required for activity and interactions with the telomerase RNA. The identified RNA–protein interactions appear to be specific for the following reasons. First, the RNA binding was inhibited with as little as 1-fold excess of unlabeled *Tetrahymena* telomerase, whereas more than a 10-fold excess of non-specific RNA was required to inhibit complex formation. Second, binding appears to be stable, because it tolerates a wide range of ionic (Kglu) concentrations and is independent of the addition of non-specific competitors such as tRNA. Although chelating agents such as

EDTA have been successfully employed to partially unfold a number of RNPs, the presence of EDTA does not appear essential to the interaction of the labeled RNA with the proteins in the extract. Dissociation of the endogenous *Tetrahymena* telomerase RNA and exchange with radiolabeled *Tetrahymena* telomerase RNA may also occur in the absence of EDTA. The optimal conditions for complex formation are ≤5 mM EDTA, 10 mM $MgCl_2$, for ≤5 min incubation at 30°C. This RNA-binding assay will be a useful tool to dissect the sequences and structures of the protein and RNA components that are essential for telomerase function.

The RNA component of telomerase has been identified from 24 ciliate species (39–41). Phylogenetic sequence comparison of these telomerase RNAs has revealed a conserved secondary structure including stem I, stem–loops II, III and IV, and a pseudoknot structure involving stem III (3,4,38,40,42). Conservation of stem–loop and pseudoknot structures is often indicative of protein-binding domains (43–46). In addition to these potential sites of protein binding, a comparison of the methylation patterns of protein-free and protein-bound *Tetrahymena* telomerase RNA has implicated several residues in protein binding. These include the hairpin loop of stem III (residues 89 and 90) adjacent to the pseudoknot, the GA bulge in stem IV (residues 121–122) and three sites around residues 15 and 16, 39 and 62 (7,8) (Fig. 1). Telomerase reconstituted *in vitro* with RNAs containing mutations in some of these residues is less active relative to telomerase reconstituted with wild-type telomerase RNA, suggesting that these residues play a role in telomerase function, perhaps indirectly, by binding to telomerase protein components (9). All of the mutated *Tetrahymena* telomerase RNAs described in this paper were tested for their ability to specifically inhibit complex formation. Some of these modified RNAs failed to inhibit complex formation as efficiently as the wild-type RNA and indicate that the deleted structures or sequences may be involved in telomerase function by directly binding to telomerase protein components. The mutated RNAs may fold differently and alternatively base pair, which may indirectly affect their binding to telomerase proteins. Secondary structure analysis of these mutated RNAs will be required to assess the different roles of RNA folding, binding and catalysis in telomere function.

Tetrahymena telomerase RNA mutants carrying deletions of 13 and residues at the 3′ end (t146 and t75, respectively) reconstitute <10% of the telomerase activity reconstituted by wild-type *Tetrahymena* telomerase RNA. This is consistent with the high sequence conservation of stem IV (3,9). The IC_{50} of t75 and non-specific *E.coli* rRNA were similar (87.8 ng for t75 versus 140.3 ng for rRNA), indicating that structures or sequences deleted in this mutant may be involved in telomerase function by binding to telomerase protein components. It is interesting that competitive inhibition of complex formation was more efficient with a mutant RNA containing a larger deletion (compare t75 to t146). Perhaps the t75 mutant RNAs forms alternate structures that are more similar to the wild-type telomerase RNA than t146.

In contrast to the 3′-modified *Tetrahymena* telomerase RNAs, a mutant carrying a deletion of 36 residues at the 5′ end (Δ5′36) was as effective as wild-type RNA in competitively inhibiting complex formation (IC_{50} = 4.7 ng for Δ5′36 versus 11.5 ng for wild-type RNA). However this RNA does not reconstitute telomerase activity *in vitro* (9). Thus, residues at the 5′ end of the RNA may be dispensable for protein binding but are essential for RNP catalysis activity. The conserved sequence 5′-(CU)GUCA-3′ (residues 35–40) upstream of the template may be engaged in an RNA–RNA or RNA–protein structure involved in preventing polymerization beyond the 5′ end of the template, and thereby define the 5′ boundary of the template (38). Studies with a mutant containing residues substituted at positions 38–40 (38–40AGU) support this hypothesis (27). Despite a role in template boundary determination, the conserved region (at least residues 38–40) does not provide significant determinants for RNA–protein interactions as this RNA inhibited the formation of the *Tetrahymena* telomerase RNA–protein complex as efficiently as the wild-type RNA (IC_{50} = 11.5 ng for wild-type versus 6.7 ng for 38–40AGU).

Tetrahymena telomerase activity cannot be reconstituted *in vitro* using *Euplotes* telomerase RNA or human telomerase RNA (C.Autexier and C.W.Greider, unpublished). Interestingly, the *Tetrahymena* telomerase RNA–protein complex was, to a limited extent, inhibited by the *Euplotes* telomerase RNA (Fig. 7). Although the *Euplotes* and *Tetrahymena* RNAs share limited primary sequence similarity, they fold into similar secondary structures, suggesting that the RNA–protein interactions that mediate complex formation in the telomerase ribonucleoprotein may involve RNA secondary structures rather than specific sequences. Differences in the apparent binding affinities of *Euplotes* and *Tetrahymena* telomerase RNA to partially purified *Tetrahymena* telomerase components may be due, in part, to differences in the protein composition of the *Euplotes* and *Tetrahymena* telomerase enzymes. The p80 and p95 homologs have not been identified in *Euplotes*, and biochemical purification of proteins associated with telomerase activity and RNA in *Euplotes aediculatus* has identified a protein, p43, not identified in any other telomerase complex to date (47).

The human and mouse telomerase RNAs are 62% identical and secondary structure predictions await phylogenetic comparisons and structural probing of these longer RNAs (37,48). Despite the higher primary sequence similarity of the mouse and human RNAs, the mouse telomerase RNA cannot reconstitute human telomerase activity (25,28). It is not surprising that *Tetrahymena* telomerase activity cannot be reconstituted with human telomerase RNA and that the human telomerase RNA did not specifically inhibit *Tetrahymena* telomerase RNA–protein complex formation (IC_{50} = 101.9 ng for hTR versus 10.3 ng for wild-type). This may be due to differences in both RNA sequence and structure between the *Tetrahymena* and human telomerase RNAs and differences in the protein composition of these telomerase complexes. Although a p80 homolog has been identified in human, it is a much larger protein of 240 kDa (11). No p95 homolog has been identified in human.

The assay for *Tetrahymena* telomerase RNA–protein binding has provided a preliminary analysis of the *Tetrahymena* telomerase RNA sequences and structures likely to be involved in stabilizing the telomerase ribonucleoprotein complex. This electrophoretic mobility shift assay will not only allow a detailed characterization of the telomerase RNA structures and sequences but will facilitate the identification and characterization of distinct telomerase protein components involved in binding to telomerase RNA.

ACKNOWLEDGEMENTS

We thank Carol Greider, in whose laboratory many of the reagents used in these experiments were generated. We thank George Kukolj, and members of the Autexier laboratory, François

Bachand and Tara Moriarty for critical review of this manuscript. We thank George Kukolj for helpful discussions during the course of the work and assistance with the statistical analysis. This work was supported by Medical Research Council of Canada grant MT-14026 to C.A.

REFERENCES

1 Greider,C.W. (1996) *Annu. Rev. Biochem.*, **65**, 337–365.
2 Nugent,C.I. and Lundblad,V. (1998) *Genes Dev.*, **12**, 1073–1085.
3 Romero,D.P. and Blackburn,E.H. (1991) *Cell*, **67**, 343–353.
4 ten Dam,E., Van Belkum,A. and Pleij,C.W.A. (1991) *Nucleic Acids Res.*, **19**, 6951.
5 Greider,C.W. and Blackburn,E.H. (1989) *Nature*, **337**, 331–337.
6 Yu,G.-L., Bradley,J.D., Attardi,L.D. and Blackburn,E.H. (1990) *Nature*, **344**, 126–132.
7 Zaug,A.J. and Cech,T.R. (1995) *RNA*, **1**, 363–374.
8 Bhattacharya,A. and Blackburn,E.H. (1994) *EMBO J.*, **13**, 5521–5531.
9 Autexier,C. and Greider,C.W. (1998) *Nucleic Acids Res.*, **26**, 787–795.
10 Collins,K., Kobayashi,R. and Greider,C.W. (1995) *Cell*, **81**, 677–686.
11 Harrington,L., McPhail,T., Mar,V., Zhou,W., Oulton,R., Bass,M.B., Arruda,I. and Robinson,M.O. (1997) *Science*, **275**, 973–977.
12 Nakayama,J., Saito,M., Nakamura,H., Matsuura,A. and Ishikawa,F. (1997) *Cell*, **88**, 875–884.
13 Gandhi,L. and Collins,K. (1998) *Genes Dev.*, **12**, 721–733.
14 Lingner,J., Hughes,T.R., Shevchenko,A., Mann,M., Lundblad,V. and Cech,T.R. (1997) *Science*, **276**, 561–567.
15 Counter,C.M., Meyerson,M., Eaton,E.N. and Weinberg,R.A. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 9202–9207.
16 Nakamura,T.M., Morin,G.B., Chapman,K.B., Weinrich,S.L., Andrews,W.H., Lingner,J., Harley,C.B. and Cech,T.R. (1997) *Science*, **277**, 955–959.
17 Meyerson,M., Counter,C.M., Eaton,E.N., Ellisen,L.W., Steiner,P., Caddle,S.D., Ziaugra,L., Beijersbergen,R.L., Davidoff,M.J., Liu,Q. *et al.* (1997) *Cell*, **90**, 785–795.
18 Harrington,L., Zhou,W., McPhail,T., Oulton,R., Yeung,D.S.K., Mar,V., Bass,M.B. and Robinson,M.O. (1997) *Genes Dev.*, **11**, 3109–3115.
19 Kilian,A., Bowtell,D.D.L., Abud,H.E., Hime,G.R., Venter,D.J., Keese,P.K., Duncan,E.L., Reddel,R.R. and Jefferson,R.A. (1997) *Hum. Mol. Genet.*, **6**, 2100–2019.
20 Greenberg,R.A., Allsopp,R.C., Chin,L., Morin,G.B. and DePinho,R.A. (1998) *Oncogene*, **16**, 1723–1730.
21 Martin-Rivera,L., Herrera,E., Albar,J.P. and Blasco,M.A. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 10471–10476.
22 Collins,K. and Gandhi,L. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 8485–8490.
23 Bryan,T.M., Sperger,J.M., Chapman,K.B. and Cech,T.R. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 8479–8484.
24 Weinrich,S.L., Pruzan,R., Ma,L., Ouellette,M., Tesmer,V.M., Holt,S.E., Bodnar,A.G., Lichtsteiner,S., Kim,N.W., Trager,J.B. *et al.* (1997) *Nature Genet.*, **17**, 498–502.
25 Beattie,T.L., Zhou,W., Robinson,M.O. and Harrington,L. (1998) *Curr. Biol.*, **8**, 177–180.
26 Autexier,C. and Greider,C.W. (1994) *Genes Dev.*, **8**, 563–575.
27 Autexier,C. and Greider,C.W. (1995) *Genes Dev.*, **15**, 2227–2239.
28 Autexier,C., Pruzan,R., Funk,W.D. and Greider,C.W. (1996) *EMBO J.*, **15**, 5928–5935.
29 Nelson,K.K. and Green,M.R. (1988) *Genes Dev.*, **2**, 319–329.
30 Blobel,G. (1971) *Proc. Natl Acad. Sci. USA*, **68**, 1881–1885.
31 Spirin,A.S. (1974) *FEBS Lett.*, **40S**, S38–S47.
32 Newton,I., Rinke,J. and Brimacombe,R. (1975) *FEBS Lett.*, **51**, 215–218.
33 Guthrie,C. and Atchison,R. (1980) In Soll,D., Abelson,J.N. and Schimmel,P.R. (eds), *Transfer RNA: Biological Aspects*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 83–97.
34 Mukherjee,A.K. and Sarkar,S. (1981) *J. Biol. Chem.*, **256**, 11301–11306.
35 Walter,P. and Blobel,G. (1983) *Cell*, **34**, 525–533.
36 Shippen-Lentz,D. and Blackburn,E.H. (1990) *Science*, **247**, 546–552.
37 Feng,J., Funk,W.D., Wang,S.S., Weinrich,S.L., Avilion,A.A., Chiu,C.P., Adams,S.S., Chang,E., Allsopp,R.C., Yu,J. *et al.* (1995) *Science*, **269**, 1236–1241.
38 Lingner,J., Hendrick,L.L. and Cech,T.R. (1994) *Genes Dev.*, **8**, 1984–1998.
39 Greider,C.W., Collins,K. and Autexier,C. (1996) In DePamphlis,M. (ed.), *DNA Replication in Eukaryotic Cells*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 619–638.
40 McCormick-Graham,M. and Romero,D.P. (1996) *Mol. Cell. Biol.*, **16**, 1871–1879.
41 Blackburn,E.H. (1998) In Simons,R.W. and Grunberg-Manago,M. (eds), *RNA Structure and Function*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 669–693.
42 McCormick-Graham,M. and Romero,D.P. (1995) *Nucleic Acids Res.*, **23**, 1091–1097.
43 Draper,D.E. (1990) In Hill,W.E., A.D., Garrett,R.A., Moore,P.B., Schlessinger,D. and Warner,J.R. (eds), *The Ribosome: Structure, Function and Evolution*. American Society of Microbiology, Washington, DC, pp. 160–167.
44 Tuerk,C., MacDougal,S. and Gold,L. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6988–6992.
45 Wyatt,J.R. and Tinoco,I.J. (1993) In Gesteland,R.F. and Atkins,J.F. (eds), *The RNA World*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 465–497.
46 Pleij,C.W.A. (1994) *Curr. Opin. Struct. Biol.*, **4**, 337–344.
47 Lingner,J. and Cech,T.R. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 10712–10717.
48 Blasco,M., Funk,W., Villeponteau,B. and Greider,C.W. (1995) *Science*, **269**, 1267–1270.