Tetrahymena proteins p80 and p95 are not core telomerase components

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Telomeres provide stability to eukaryotic chromosomes and consist of tandem DNA repeat sequences. Telomeric repeats are synthesized and maintained by a specialized reverse transcriptase, termed telomerase. *Tetrahymena thermophila* telomerase contains two essential components: *Tetrahymena* telomerase reverse transcriptase (tTERT), the catalytic protein component, and telomerase RNA that provides the template for telomere repeat synthesis. In addition to these two components, two proteins, p80 and p95, were previously found to copurify with telomerase activity and to interact with tTERT and telomerase RNA. To investigate the role of p80 and p95 in the telomerase enzyme, we tested the interaction of p80, p95, and tTERT in several different recombinant expression systems and in *Tetrahymena* extracts. Immunoprecipitation of recombinant proteins showed that although p80 and p95 associated with each other, they did not associate with tTERT. In *in vitro* transcription and translation lysates, tTERT was associated with telomerase activity, but p80 and p95 were not. p80 bound telomerase RNA as well as several other unrelated RNAs, suggesting p80 has a general affinity for RNA. Immunoprecipitations from *Tetrahymena* extracts also showed no evidence for an interaction between the core tTERT/telomerase RNA complex and the p80 and p95 proteins. These data suggest that p80 and p95 are not associated with the bulk of active telomerase in *Tetrahymena*.

Telomeres provide stability to chromosomes by preventing end degradation and end-to-end chromosome fusions (reviewed in refs. 1 and 2). Telomeric DNA consists of repetitive GT-rich sequences that are conserved in most eukaryotic organisms. These DNA repeats are synthesized and maintained by the specialized reverse transcriptase telomerase (reviewed in ref. 3). Telomerase enzyme activity was first identified in the ciliate *Tetrahymena thermophila* (4). The enzyme was later found to contain an essential RNA component that provides the template for telomere repeat synthesis (5). Telomerase RNA components have now been identified from many other organisms including ciliates, yeasts, and vertebrates (6–15). The catalytic protein component, termed telomerase reverse transcriptase (TERT), was first discovered in the ciliate *Euplotes audiculatus* (16). Homologues have been found in yeast, mammals, plants, ciliates, and *Caenorhabditis elegans* (17–27). Both human and *Tetrahymena* telomerase enzyme activity can be reconstituted in an *in vitro* transcription and translation reaction in rabbit reticulocyte lysates expressing TERT in the presence of telomerase RNA (25, 28, 29). Human telomerase activity also has been reconstituted in yeast by coexpressing human TERT (hTERT) and human telomerase RNA and by the addition of human telomerase RNA to recombinant hTERT expressed in insect cells (30, 31). Thus, these two components are the minimal core components of the telomerase enzyme.

In addition to the core telomerase components, several other proteins have been found to be associated with telomerase. In yeast, *EST1* and *EST3* interact with telomerase *in vivo* and *in vitro* (32, 33). Deletion of each of these genes or the genes encoding TERT or telomerase RNA results in the identical telomere shortening phenotype, indicating *EST1* and *EST3* are essential for telomerase action *in vivo* (32). However, telomerase activity is still present in cells that lack these genes (34). *EST1* and *EST3* physically interact with telomerase and telomerase RNA, indicating they are telomerase-associated proteins (33). In human cells, telomerase-associated protein 1 (TEP1) and the chaperone proteins hsp90 and p23 were found to interact with the hTERT protein and telomerase activity (35, 36). The proteins L22, hStau, and dyskerin bind human telomerase RNA and are associated with telomerase activity in cell extracts (37, 38). In the ciliate *E. audiculatus,* the protein p43 was identified by copurification with TERT and telomerase activity (39). p43 is physically associated with telomerase activity in cell extracts and is a homologue of the human La protein (40).

In *Tetrahymena*, p80 and p95 were identified by copurification with telomerase enzyme activity and telomerase RNA (41). These two proteins tightly interact with each other and were reported to bind to telomerase RNA and *Tetrahymena* TERT (tTERT) in *Tetrahymena* extracts (25). Purified recombinant p80 and p95 proteins were shown to bind to *in vitro*-transcribed telomerase RNA, and p95 bound to telomeric oligonucleotides, suggesting these proteins are integral components of telomerase (42). However, deletion of these proteins in *Tetrahymena* did not affect the levels of telomerase RNA or telomerase activity (43).

A mammalian telomerase protein, TEP1, was identified by homology to the p80 protein from *Tetrahymena* (35, 44). TEP1 was shown to associate with telomerase activity, telomerase RNA, and hTERT in human cell extracts. TEP1 has an amino-terminal region of homology to p80 and a large carboxyl-terminal domain with 12 WD repeat motifs (35). In addition to its association with telomerase, TEP1 is a component of the cytoplasmic particles termed vaults. Vaults are ribonucleoprotein (RNP) complexes that contain a small RNA, which binds to TEP1 (45). The role of TEP1 for telomerase function is not clear, as the absence of TEP1 does not effect telomerase activity or telomere length in mice (46).

To understand the role of telomerase-associated proteins, we set out to characterize the interaction of p80 and p95 with *Tetrahymena* telomerase. Our analysis of recombinant proteins suggests that p80 and p95 form a complex with each other but do not associate with the catalytic component tTERT *in vitro*. We further show that in *Tetrahymena* extracts there is no measurable interaction of these proteins with telomerase, suggesting that they are not associated with the bulk of active telomerase.

Materials and Methods

Gene Construction. The *Tetrahymena* genetic code differs from other eukaryotes (47). To express p80 and p95, the synthetic genes were redesigned to convert the UAG and UAA (stop)

Abbreviations: RNP, ribonucleoprotein; TERT, telomerase reverse transcriptase; tTERT, *Tetrahymena* TERT; hTERT, human TERT; TEP1, telomerase-associated protein 1.

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codons that encode glutamine in *Tetrahymena* to the appropriate CAG codon. Overlapping oligonucleotides of ≈100 bases were synthesized (Bioserve Biotechnologies, Laurel, MD). These oligonucleotides were designed with convenient restriction enzyme sites for subsequent cloning. Overlapping oligonucleotides were annealed and extended by PCR to create a series of overlapping DNA fragments. These overlapping fragments were annealed and extended further by PCR to create 800–1,000 base-pair fragments. The fragments were then cloned into pBluescript (Stratagene) and pSE280 (Invitrogen) vectors for p80 and p95, respectively. Any point mutations or small deletions were corrected by site-directed mutagenesis (CLONTECH). The tTERT gene was generated by a similar procedure and was a kind gift from Kathleen Collins, University of California, Berkeley (25).

***Escherichia coli* Protein Expression and Purification.** p80 was tagged at the amino terminus with six histidines (6His) and was expressed from the plasmid pQE30 (Qiagen, Chatsworth, CA) in TG1 bacteria. p95 and tTERT were also tagged with six histidines at their amino termini and expressed from the plasmid pBAD/HisA in LMG194 bacterial strain (Invitrogen). To express p80 and p95 as untagged recombinant proteins, the genes were cloned into pBAD/HisA followed by the removal of the 6His tag sequence. Colonies were initially grown overnight in 5 ml of LB at 37°C and then inoculated into 50 ml of LB and grown to OD = 0.6 at 37°C. Expression was induced with 100 μM isopropyl β-D-thiogalactoside (pQE30) or 0.02% (pBAD) arabinose, and cells were grown at 23°C for 2–3 h. Cells were harvested in 50 mM NaH_2PO_4 (pH 8.0)/300 mM NaCl/10 mM imidazole/0.5% Triton X-100 with 10 μg/ml leupeptin and 0.1 mM PMSF. Cell suspensions were incubated with 1 mg/ml lysozyme for 30 min on ice. Lysates were sonicated and centrifuged at 10,000 $\times$ *g* for 30 min at 4°C. Purification of 6His-tagged proteins with Ni^{2+}-NTA resin was performed as described by the manufacturer (Qiagen). Fractions were analyzed by SDS/7.5% PAGE and stained with Coomassie blue. Peak fractions were concentrated with Centricon spin columns (Amicon).

Antibodies and Immunoprecipitations. The p80-R and p95-R antibodies were raised against the purified 6His-tagged recombinant proteins. p80-P and p95-P antibodies were raised against synthesized peptides from the p80 sequence CRKKTMFRYLSVTNKQKWDQTKKKRKEN and p95 sequence CGTYYDYNSDRW. TERT-P antibody was raised against a peptide from the carboxyl terminus of the protein: CNNLIQDIKTLIPKISAKSNQQNTN. All immunogens were conjugated to keyhole limpet hemacyanin and injected into rabbits (Convance, Denver, PA). The H1 antibody was raised against *Tetrahymena* linker histone protein H1 and was obtained from the laboratory of David Allis, University of Virginia, Charlottesville. For immunoprecipitations from *E. coli*, Sf9 insect cell extracts, and rabbit reticulocyte lysates, 1–2 μl of antisera was prebound to protein A Sepharose beads (Amersham Pharmacia) and washed in PBS. For immunoprecipitation from *Tetrahymena* extracts, antibodies were cross-linked to protein A beads as described in the Immunopure protein A IgG orientation kit (Pierce). Use of cross-linked antibody significantly reduced background during Western analysis. The antibody beads were incubated with extracts containing 1 mg of total protein at 4°C for 2 h. Beads were washed four times with the buffer each extract was made in and resuspended in SDS loading buffer. Elution of antigens from the cross-linked antibodies was performed with 0.1 M glycine (pH 2.8). Samples eluted at low pH were neutralized with final concentration of 30 mM Tris (pH 9.0) before resuspending in SDS loading buffer. All antibodies have similar immunoprecipitation efficiencies of ≈50% based on immunoprecipitations of proteins from rabbit reticulocyte lysates.

Western Analysis and Autoradiography. After SDS/PAGE, proteins were transferred to Immobilon-P poly(vinylidene difluoride) membrane (Millipore) in 48 mM Tris/39 mM glycine/20% methanol by using a semidry transfer apparatus at 15 V for 30 min (Bio-Rad). The membrane was blocked with 5% dry milk in TBS/0.1% Tween 20. The primary antibodies p80-R and p95-R were used at 1:10,000 dilution, and TERT-P antibody was used at 1:5,000 dilution in 5% dry milk in TBS/0.1% Tween 20 and incubated with blocked membrane. Goat anti-rabbit conjugated to horseradish peroxidase (Bio-Rad) was used at 1:5,000 dilution followed by enhanced chemiluminescence (Amersham Pharmacia). Protein gels to be detected by autoradiography were fixed with 50% methanol and 10% acetic acid for 30 min and then soaked in fluorographic reagent for 15 min (Amersham Pharmacia). Gels were dried and exposed to Hyperfilm (Amersham Pharmacia).

Baculovirus Expression. p80, p95, and tTERT were cloned into pFastBac1 or pFastBacHT vectors, and the protocol described in the Bac-to-Bac expression system was followed (GIBCO/BRL). To produce protein from the recombinant viruses, ≈25 μl of high titer virus was used for infection of 2.0×10^6 Sf-9 insect cells grown in Grace's insect cell culture medium with 10% FBS. After infection for 4–5 days with recombinant baculovirus, cells were harvested and lysed with 50 mM NaH_2PO_4, pH 8.0/300 mM NaCl/0.5% Triton X-100/10 μg/ml leupeptin/0.1 mM PMSF. Lysates were centrifuged at 10,000 $\times$ *g* for 30 min at 4°C and then used in immunoprecipitation reactions.

***In Vitro* Transcription and Translation Reactions.** p80, p95, and tTERT were expressed from pCITE-4a (Novagen) by using the TNT quick coupled transcription/translation system (Promega). *In vitro*-transcribed telomerase RNA was added or was expressed in the lysate from a PCR product containing the telomerase RNA gene behind the T7 promoter. Both coexpression of tTERT and telomerase RNA or telomerase RNA addition at the beginning of the transcription and translation reaction reconstituted the same level of telomerase activity. Lysates were diluted 8-fold with 10 mM Hepes, pH 7.9/100 mM potassium glutamate/1 mM $MgCl_2$/10% glycerol/0.5 mg/ml BSA/0.1 mg/ml yeast tRNA/1 mM DTT. Lysates were centrifuged at 10,000 $\times$ *g* to remove particulate matter before immunoprecipitation.

***In Vitro* RNA Transcription.** Telomerase RNA (5) was *in vitro*-transcribed as described by the manufacturer (GIBCO/BRL) by T7 RNA polymerase from a PCR product containing the gene encoding the telomerase RNA behind the T7 promoter and gel-purified on 4% PAGE. Full-length 7SL and U2 RNA DNA templates for T7 transcription in rabbit reticulocyte lysates were created by PCR from *Tetrahymena* genomic DNA. A random RNA sequence for T7 transcription was generated from a PCR template derived from the plasmid pUC19 (CLONTECH). All *in vitro* transcripts contain three extra G residues at the 5′ end.

***Tetrahymena* Extracts.** Strains CU428 and B2086 were grown to midlog phase, starved, and mated as described in ref. 41. Cells were lysed with 10 mM Hepes, pH 7.9/10 mM KCl/1 mM $MgCl_2$/10% glycerol/0.2% Nonidet P-40 and protease inhibitor mixture (Roche Molecular Biochemicals) and centrifuged at 10,000 $\times$ *g* at 4°C for 30 min.

Telomerase Assays. Telomerase assays were performed by using 3 μl of *in vitro* transcription and translation reaction lysate or 20 μl of beads containing immobilized protein in immunoprecipitation wash buffer. Samples were added to a telomerase reaction mix that contained 0.6 μM primer $d(TTGGGG)_3$/250 μM dTTP/0.85 μM dGTP/0.17 μM [α-^{32}P]dGTP (3,000 Ci/mmol)/

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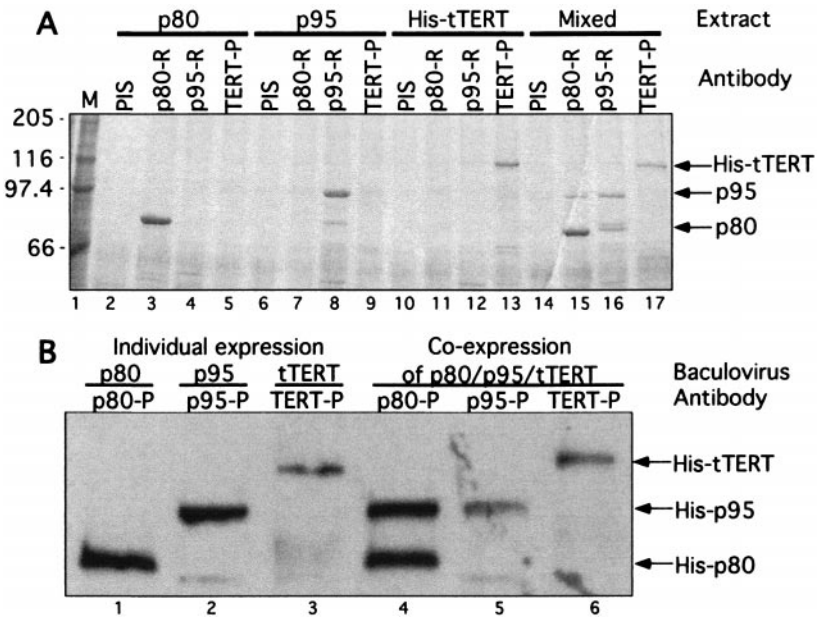

Fig. 1. (*A*) *In vitro* interactions of recombinant p80, p95, and tTERT in *E. coli* extracts. Immunoprecipitation reactions were done from *E. coli* extracts that contain p80, p95, and tTERT (lanes 2–13) or from a mixed extract containing all three proteins (lanes 14–17). The samples were resolved by SDS/PAGE and stained with Coomassie blue. The p80 and p95 recombinant proteins were expressed without any epitope tags, whereas the tTERT protein had an amino-terminal 6His tag. The extract used for each set of immunoprecipitations is indicated at the top, and the antibodies used are indicated at the top of each lane. PIS, preimmune serum. Molecular mass marker was loaded in lane 1 with indicated masses in kDa to the left. (*B*) *In vitro* interactions of recombinant p80, p95, and tTERT in baculovirus-infected insect cells. p80, p95, and tTERT were expressed individually or together in Sf9 insect cells by baculovirus infection. All three proteins were expressed with an amino-terminal 6His tag. Immunoprecipitations were performed from cells that express individual components (lanes 1–3) or those that coexpressed p80, p95, and tTERT (lanes 4–6). Proteins were detected by Western analysis with antibodies against all three proteins.

1.5 mM $MgCl_2$/50 mM Tris acetate (pH 8.5)/1 mM spermidine/5 mM β-mercaptoethanol/60 mM potassium acetate. Samples were assayed in a final volume of 40 μl at 30°C for 60 min. The reactions were phenol/chloroform-extracted, precipitated, and resolved on 8% (19:1) acrylamide/7 M urea/0.6 × TBE (Tris/boric acid/EDTA) gel. Dried gels were exposed to phosphorimager plates (Fuji) or x-ray film (Kodak).

Northern Analysis. RNA was extracted from immunoprecipitation reactions by acid phenol/chloroform and precipitated. RNA was then resolved on 6% (19:1) acrylamide/7 M urea/1 × TBE gels and transferred to Hybond-N+ membrane (Amersham Pharmacia) for 45 min at 1 amp by using an electroblotter. RNA was UV-cross-linked to the membrane with a UV stratalinker (Stratagene) and then was subjected to hybridization in 200 mM Na_2HPO_4, pH 7.2/15% formamide/7% SDS/1 mM EDTA/1% BSA at 63°C. RNA was detected with random hexamer radiolabeled DNA probes as described (48). The membrane was then washed four times with 6 × SSC and 0.1% SDS at 63°C and exposed to phosphorimager plates (Fuji) or x-ray film (Kodak).

Results and Discussion

To examine the interactions of p80, p95, tTERT, and the telomerase RNA, we expressed the four components as recombinant products and assayed their interactions by using several different systems. To express the p80, p95, and tTERT proteins in heterologous systems, the codons were redesigned to conform to the standard eukaryotic genetic code (see *Materials and Methods*). Antibodies were raised against both p80- and p95-derived synthetic peptides and recombinant proteins. Antibodies also were raised against a peptide from the carboxyl-terminal region of tTERT (see *Materials and Methods*). Coimmunoprecipitation experiments were first carried out by using *E. coli* and baculovirus systems to test protein–protein interactions in the absence of telomerase RNA. The role of the telomerase RNA in the complex was then tested by using *in vitro* transcription and translation lysates as well as extracts from *Tetrahymena* cells.

p80, p95, and tTERT Interaction in *E. coli* Extracts. To test for protein–protein interactions, p80 and p95 were expressed as untagged proteins, and the tTERT was expressed as a 6His-tagged fusion protein. Immunoprecipitations were performed on extracts containing one of the proteins or from mixed extracts containing all three proteins. The pellets from the immunoprecipitation reaction were then resolved by SDS/PAGE and visualized with Coomassie blue (Fig. 1*A*). Each polyclonal antibody specifically immunoprecipitated the protein it was raised against. Preimmune serum did not immunoprecipitate any of the three recombinant proteins. Both the p80 and p95 antibodies raised against recombinant protein were able to coimmunoprecipitate a complex containing both p80 and p95, indicating that these proteins associate with each other as described (42). However, the tTERT antibody only immunoprecipitated tTERT, suggesting that there was no interaction between the p80/p95 complex and tTERT in this recombinant system.

p80, p95, and tTERT Interactions in Insect Cell Extracts. There are several reasons we might have failed to detect an association of tTERT with p80 and p95 in the *E. coli* system. First, the proteins might require a posttranslational modification not generated in *E. coli*. Second, the proteins may need to be expressed and translated in the same cell to allow complex formation. To address these issues, we expressed the three proteins in Sf9 insect cells by using the baculovirus expression system (see *Materials and Methods*). All three proteins (p80, p95, and tTERT) were expressed as 6His-tagged fusion proteins. The proteins were either expressed alone or coexpressed by virus coinfection to allow for the assembly of potential protein complexes within the insect cells. Western analysis confirmed the expression of each protein from insect cells expressing individual proteins or coexpressing p80, p95, and tTERT (data not shown). The immunoprecipitation reactions were carried out with p80 and p95 antibodies that were raised against synthetic peptides rather than those raised against recombinant proteins. The pellets from these immunoprecipitation reactions were resolved by SDS/PAGE, and proteins were detected by Western analysis with antibodies against p80, p95, and tTERT (Fig. 1*B*). All three antibodies were able to immunoprecipitate the corresponding target protein. As observed earlier, the p80 antipeptide antibody coimmunoprecipitated both p80 and p95 from the extracts made from coinfected cells. The p95 antipeptide antibody immunoprecipitated only p95 and not p80. Failure of this antibody to immunoprecipitate the p80/p95 complex may result from inac-

cessibility of the specific epitope in the p80/p95 complex. As shown above, polyclonal antibodies raised against the p95 recombinant protein did immunoprecipitate the p80/p95 complex (Fig. 1*A*). The antipeptide antibody against tTERT only immunoprecipitated tTERT and did not coimmunoprecipitate the p80/p95 complex. Again, these immunoprecipitations failed to show an interaction of p80/p95 with tTERT. The tTERT peptide antibody used in these immunoprecipitation experiments may have not detected a potential complex if its specific epitopes were masked. To test this, we used the 6His tag placed at the amino terminus of each protein to purify potential complexes in both the *E. coli* and baculovirus expression systems. Again, no interaction between p80/p95 and tTERT was seen when each protein was purified by its specific amino-terminal tag (data not shown).

p80, p95, tTERT, and Telomerase RNA Interactions in Rabbit Reticulocyte Lysates. An important component of the telomerase complex that was missing in these experiments is the telomerase RNA. To test whether the telomerase RNA is needed for the association of p80/p95 with tTERT, we used an *in vitro* transcription and translation reaction in rabbit reticulocyte lysates to express p80, p95, tTERT, and telomerase RNA. Because telomerase activity can be reconstituted in *in vitro* transcription and translation lysates (25, 28, 29), we examined the association of each component with active telomerase. The three protein components were coexpressed in *in vitro* transcription and translation lysates containing ^{35}S-radiolabeled methionine. Telomerase RNA was either coexpressed or added at the beginning of the transcription and translation reaction. Immunoprecipitations from these reactions were assayed for protein interactions, telomerase RNA binding, and telomerase activity (Fig. 2). Analysis of the *in vitro* transcription and translation reaction before immunoprecipitation showed that all three proteins, telomerase RNA, and telomerase activity were present. Preimmune serum did not immunoprecipitate p80, p95, tTERT, telomerase RNA, or telomerase activity. Antibodies raised against recombinant p80 and p95 coimmunoprecipitated the p80/p95 protein complex but not tTERT or telomerase activity. However, telomerase RNA did coimmunoprecipitate with the p80/p95 complex. The tTERT antibody immunoprecipitated tTERT, telomerase RNA, and telomerase activity but not the p80/p95 complex. This suggested that p80 and p95 are not a part of an active telomerase complex but are capable of interacting with the telomerase RNA subunit as has been described (42). Finally, we assayed telomerase activity in crude transcription and translation lysates before immunoprecipitation. The presence of p80 and/or p95 with tTERT and telomerase RNA affected neither the telomerase activity level nor the length or pattern of the products produced (data not shown).

To determine whether p80, p95, or both proteins were mediating the interaction with telomerase RNA, each protein was individually expressed in the *in vitro* transcription and translation reaction. Results of immunoprecipitations showed that telomerase RNA was precipitated only in the reactions containing p80, indicating that this is the subunit that associates with telomerase RNA (data not shown). To address the specificity of telomerase RNA binding, the amount of telomerase RNA added to *in vitro* transcription and translation reactions was titrated to limiting amounts. Telomerase RNA binding was assayed by immunoprecipitation followed by Northern analysis (Fig. 3*A*). At low levels of input RNA, tTERT bound a significant amount of telomerase RNA but the p80/p95 complex did not, suggesting that tTERT has a higher specificity for telomerase RNA than p80. Autoradiography showed equal amounts of p80, p95, and tTERT were immunoprecipitated (Fig. 3*B*).

p80 and its human homologue TEP1 were previously shown to bind telomerase RNA and vault RNA (45). To further test the specificity of the p80 interaction with telomerase RNA, we

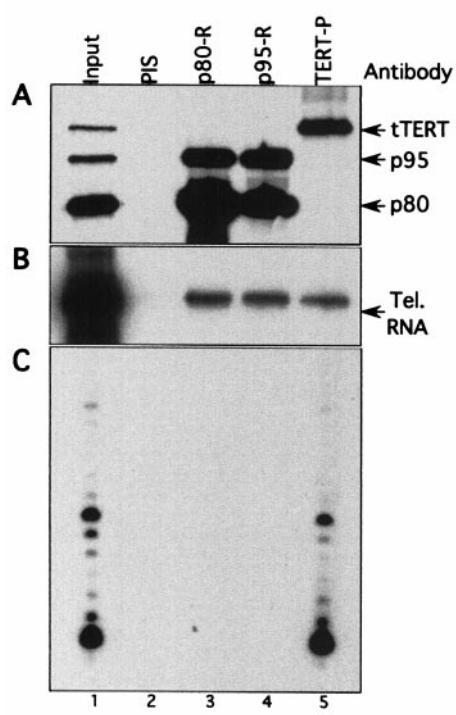

Fig. 2. Interactions of recombinant p80, p95, and tTERT and telomerase RNA in rabbit reticulocyte lysates. p80, p95, tTERT, and telomerase RNA were expressed by *in vitro* transcription and translation in the presence of [^{35}S]methionine by using rabbit reticulocyte lysates. The input material is shown in lane 1. Immunoprecipitations were performed with the antibodies indicated at the top of each lane (lanes 2–5). (*A*) Autoradiography of expressed and immunoprecipitated ^{35}S-methionine-labeled proteins after SDS/PAGE. PIS, preimmune serum. (*B*) Northern analysis to detect telomerase RNA from immunoprecipitations. (*C*) Telomerase enzyme activity from immunoprecipitations.

examined the interaction of p80 with several other unrelated RNAs. *Tetrahymena* 7SL, U2, and a random RNA sequence were included along with telomerase RNA in the *in vitro* transcription and translation reaction, and immunoprecipitations were carried out. All four RNAs were immunoprecipitated with antibodies against p80; however, only the telomerase RNA was immunoprecipitated with the antibodies against tTERT (Fig. 4 *A* and *B*). Autoradiography showed that the appropriate proteins were immunoprecipitated in these reactions (Fig. 4*C*). Thus, the association of p80 with telomerase RNA could be due to its nonspecific interaction with a variety of RNAs.

p80, p95, tTERT, and Telomerase *in Vivo* Interactions in *Tetrahymena*. To investigate whether the interaction of p80 and p95 with tTERT and telomerase RNA might require some additional factor, we carried out immunoprecipitation experiments directly from *Tetrahymena* extracts. Because of the low tTERT levels in *Tetrahymena*, antibodies were first cross-linked to protein A beads before immunoprecipitation (see *Materials and Methods*) and then detected by Western analysis (Fig. 5*A*). A titration of all three purified recombinant proteins probed by Western analysis showed very similar signals for p80, p95, and tTERT, indicating that the affinity of the antibodies is equivalent (data not shown). In addition to Western analysis, the immunoprecipitation reactions were assayed for telomerase RNA and telomerase activity (Fig. 5 *B* and *C*). Preimmune serum and a control antibody against *Tetrahymena* histone protein (H1) did not immunoprecipitate p80, p95, tTERT, or telomerase RNA, and only background levels of telomerase activity were observed.

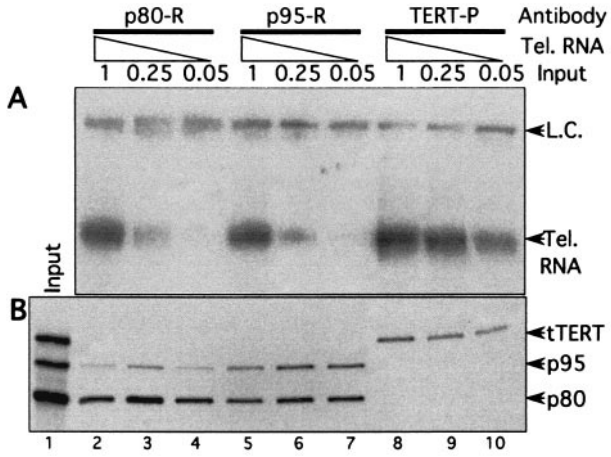

Fig. 3. Specificity of telomerase RNA protein binding. p80, p95, and tTERT were expressed by *in vitro* transcription and translation in the presence of [^{35}S]methionine using rabbit reticulocyte lysates. After 1 h of protein synthesis, telomerase RNA was added to a final concentration of 1.0, 0.25, or 0.05 ng/μl and allowed to bind for 30 min. Immunoprecipitations were performed with the p80-R (lanes 2–4), p95-R (lanes 5–7), and TERT-P (lanes 8–10) antibodies from each reaction. (*A*) Northern analysis to detect telomerase RNA from immunoprecipitations. A DNA loading control (L.C.) was added before RNA extraction. (*B*) Autoradiography of immunoprecipitated ^{35}S-methionine-labeled proteins after SDS/PAGE (lanes 2–10). Input lysate after 1 h of protein synthesis before telomerase RNA addition is shown in lane 1.

Antibodies against p80 and p95 coimmunoprecipitated the p80/p95 complex but not tTERT, telomerase RNA, or significant levels of telomerase activity. The tTERT antibody immunoprecipitated tTERT, telomerase RNA, and high levels of telomerase activity but not the p80/p95 complex. The tTERT antibody also immunoprecipitated a tTERT degradation product, which was also observed in baculovirus-infected insect cell extracts and in *in vitro* transcription and translation lysates. As an additional control, we preincubated the tTERT antibody with the peptide against which the antibody was raised. This blocked precipitation of tTERT, telomerase RNA, and telomerase activity. This evidence suggested that the p80/p95 complex is not associated with the bulk of the tTERT/telomerase RNA complex in *Tetrahymena* cell extracts.

In the ciliate *Euplotes crassus,* telomerase activity fractionates into several higher-order complexes during development (49).

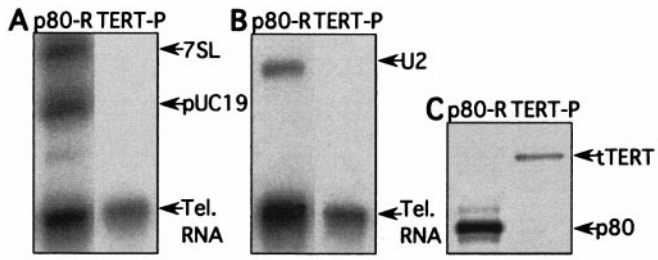

Fig. 4. Interactions of p80 with various RNAs. p80 and tTERT were expressed individually by *in vitro* transcription and translation in the presence of [^{35}S]methionine using rabbit reticulocyte lysates. DNA templates for T7 transcription of telomerase RNA, 7SL RNA, U2 RNA, and a random RNA derived from pUC19 were included in the reaction. Immunoprecipitations were performed with the p80-R and TERT-P antibodies. (*A*) Northern analysis to detect telomerase RNA, 7SL RNA, and random RNA from immunoprecipitations. (*B*) Northern analysis to detect telomerase RNA and U2 RNA from immunoprecipitations. (*C*) Autoradiography of immunoprecipitated ^{35}S-methionine-labeled proteins after SDS/PAGE.

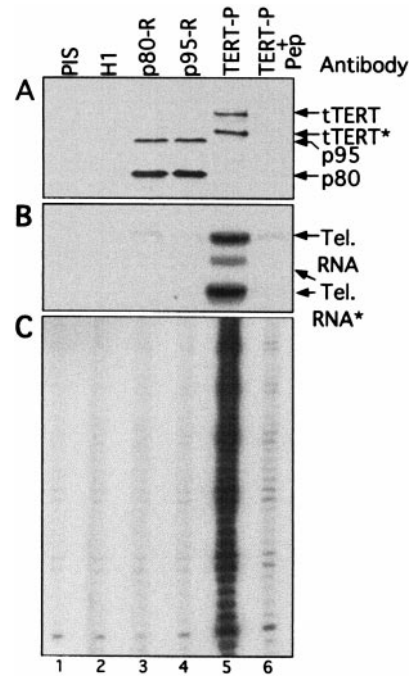

Fig. 5. Interactions of p80, p95, tTERT, and telomerase RNA in *Tetrahymena* extracts. Immunoprecipitations from *Tetrahymena* extracts were done with the antibodies indicated at the top of each lane (lanes 1–5). PIS, preimmune serum. The H1 antibody was raised against *Tetrahymena* linker histone protein H1. Antibodies were cross-linked to protein A beads (see *Materials and Methods*). The TERT-P antibody was also prebound with the carboxyl-terminal tTERT peptide, against which it was raised before the addition to the extract (lane 6). (*A*) Western analysis with antibodies against p80, p95, and tTERT of proteins eluted from cross-linked antibody immunoprecipitation pellets. tTERT* indicates a degradation product. (*B*) Northern analysis to detect telomerase RNA from immunoprecipitations. Telomerase RNA* indicates a degradation product. (*C*) Telomerase enzyme activity from immunoprecipitations.

To examine whether the p80/p95 complex is found associated with telomerase at a particular developmental stage, we tested the association of the components in both mated and vegetative extracts. No association was detected in either type of extract (data not shown). It remains possible that p80 and p95 are associated with the telomerase core enzyme during a particular stage in the *Tetrahymena* life cycle.

The complexes seen in *Tetrahymena* extracts were similar to those observed in the *in vitro* transcription and translation reactions except the p80/p95 complex did not interact with telomerase RNA. Similarly, the p80/p95 complex did not interact with *Tetrahymena* 7SL and U2 RNA in *Tetrahymena* extracts (data not shown). Thus, the specificity of RNA–protein interactions is significantly greater *in vivo* than *in vitro*. Perhaps p80 is in a specific cellular compartment and/or bound to a specific RNA and is not free to exhibit nonspecific RNA interactions *in vivo*.

Telomerase associated-proteins have a variety of functions such as recruitment of telomerase to the telomere, RNA stability, RNP assembly, and localization. In yeast, *EST1*, *EST3*, and *CDC13* are not required for telomerase enzyme activity but are essential for telomere maintenance *in vivo* (32, 34). In ciliates, the only other identified telomerase-associated protein is p43 from *E. audiculatus* (40). This protein shares homology to the human La protein that has been implicated in several functions such as RNA termination and RNP maturation of RNA polymerase III transcripts (reviewed in ref. 50). p43 likely associates with telomerase because in ciliates telomerase RNA is a RNA polymerase III transcript (51). Whether p80 and p95 play any

role in *Tetrahymena* telomerase is unclear. *Tetrahymena* strains lacking p80 and p95 do not show any changes in telomerase activity or in the levels of telomerase RNA. Loss of p95 affects the resetting of telomere length during *Tetrahymena* development; however, it is not clear whether this is a direct effect (43). Mice that are deficient for the p80 homologue TEP1 also do not show any changes in telomerase activity or telomere length (46). Although TEP1 can associate with telomerase, it is predominantly found as a component of the abundant vault particles (45).

The affinity of p80 for RNA suggests that the *Tetrahymena* p80/p95 complex may also be an RNP. The biochemical properties of this RNP may be similar to the TERT/telomerase RNA complex and thus explain why the cellular p80/p95 complex copurified with telomerase activity over a variety of columns (41). Our current studies showing the lack of association of p80 and p95 with active telomerase both *in vivo* and *in vitro* suggest that these proteins are not integral components of the telomerase enzyme complex.

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