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Purification of Tetrahymena Telomerase and Cloning of Genes Encoding the Two Protein Components of the Enzyme

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Summary

Telomerase is a ribonucleoprotein DNA polymerase that catalyzes the de novo synthesis of telomeric simple sequence repeats. We describe the purification of telomerase and the cloning of cDNAs encoding two protein subunits from the ciliate Tetrahymena. Two proteins of 80 and 95 kDa copurified and coimmunoprecipitated with telomerase activity and the previously identified Tetrahymena telomerase RNA. The p95 subunit specifically cross-linked to a radiolabeled telomeric DNA primer, while the p80 subunit specifically bound to radiolabeled telomerase RNA. At the primary sequence level, the two telomerase proteins share only limited homologies with other polymerases and polymerase accessory factors.

Introduction

The faithful replication of eukaryotic chromosomes requires the activity of several DNA-dependent DNA polymerases, a DNA-dependent RNA polymerase (primase), and the RNA-dependent DNA polymerase telomerase. Replication by DNA-dependent polymerases alone does not completely duplicate the sequences at chromosome termini, resulting in a loss of telomeric simple sequence repeats with each cell division. Telomerase compensates for this underreplication by de novo addition of simple sequence repeats (for review, see Greider, 1991). Conditions that affect the balance in number of telomeric simple sequence repeats also affect chromosome stability, gene expression, and likely additional cellular processes (for review, see Blackburn, 1994). Primary human cell cultures that exhibit a loss of telomeric simple sequence repeats with proliferation do not possess detectable telomerase activity (Counter et al., 1992). These cells become senescent after the number of population doublings required for shortening of telomeric simple sequence repeat tracts to a predicted critical minimum size, independent of initial length (Counter et al., 1992; Allsopp et al., 1992). Thus, telomeric simple sequence repeats have been proposed to act as a molecular clock for proliferation, counting down a set number of cell divisions. Many cancer cells, unlike their somatic progenitors, possess telomerase activity (Counter et al., 1994; Kim et al., 1994). If telomerase activation is a requirement for the continued growth of cancer cells, it would provide a potential target for therapeutic treatment (Harley et al., 1994).

Telomerase is unique among eukaryotic polymerases in that it functions as a stable ribonucleoprotein (RNP) complex. The 159 nt Tetrahymena thermophila telomerase RNA component contains a 9 nt sequence (3′-AACCCC-AAC-5′) complementary to the 6 nt telomeric repeat, d(TTGGGG) (Greider and Blackburn, 1989). This region of the telomerase RNA templates the synthesis of telomeres in vivo (Yu et al., 1990). Additional telomerase RNAs have been cloned from both ciliates and yeast (Shippen-Lentz and Blackburn, 1990; Romero and Blackburn, 1991; Lingner et al., 1994; Melek et al., 1994; Singer and Gottschling, 1994). Aside from the presence of a template region, these RNAs lack extensive primary sequence conservation. Phylogenetic evidence suggests a conserved secondary structure in the ciliate RNAs (Lingner et al., 1994; Romero and Blackburn, 1991), but the function of the conserved structures is not yet known.

Models for primer recognition by Tetrahymena telomerase have proposed two primer-binding sites to account for the differences in elongation processivity and binding affinity of different primers (see, for example, Collins and Greider, 1993; Lee and Blackburn, 1993). During processive elongation, the product 3′ end is released from the end of the template and repositioned at the beginning of the template to allow addition of another repeat to the same substrate. When released from the template, product remains associated with telomerase by interaction at a second site (the anchor site). Binding at the second site is less sequence specific than binding of primer at the template site; however, telomeric or G-rich sequences are preferred (Collins and Greider, 1993; Lee and Blackburn, 1993). Telomerase also catalyzes a nucleolytic cleavage activity. When primer or product is aligned at the extreme 5′ end of the RNA template, residues at the 3′ end of the substrate can be removed (Collins and Greider, 1993). The processive elongation and nucleolytic cleavage catalyzed by telomerase are similar to activities catalyzed by DNA-dependent RNA polymerases: in both cases, the 3′ end of the product, labile to nucleolytic cleavage, is bound at the template or active site, while a more 5′ region of product is bound at a second site on the enzyme (for RNA polymerase, see Kassavetis and Geiduschek, 1993). To analyze telomerase in molecular detail, it was essential to identify and characterize the protein components of the enzyme. In this report, we describe the purification of Tetrahymena telomerase and the cloning of cDNAs encoding the two protein subunits of this novel RNP polymerase.

Results

Purification of the Tetrahymena Telomerase RNP

Optimal purification of telomerase was obtained by chromatography of a cytoplasmic extract on hydroxylapatite, spermine–agarose, Sepharose CL-6B, phenyl–Sepharose, and DEAE–agarose or Q-Sepharose resins, followed by glycerol gradient sedimentation (see Experimental Procedures). Telomerase activity was assayed and the telomerase RNA component quantitated with each fractionation step. Telomerase activity assayed across a representative glycerol gradient is shown in Figure 1A. Activity was con-

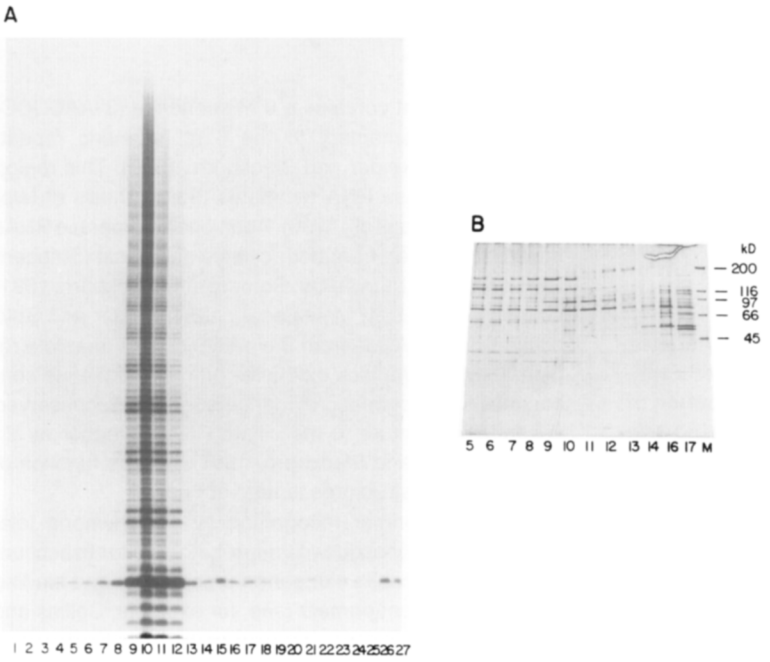

Figure 1. Purification of Tetrahymena Telomerase

(A) Telomerase activity across glycerol gradient fractions. The indicated fractions were assayed for telomerase activity. The bottom of the gradient is fraction 1.

(B) Copurification of p95 and p80 with telomerase activity. The indicated fractions were analyzed by SDS–PAGE and silver staining. The M lane contains markers of the indicated molecular masses.

centrated in fractions 9–12 and peaked in fraction 10. The distribution of telomerase RNA coincided exactly with telomerase activity across the gradient (data not shown). Two proteins cofractionated with telomerase activity and RNA (Figure 1B), migrating at 95 kDa (p95) and 80 kDa (p80). In this gradient, more than one 80 kDa polypeptide was present in the fractions containing telomerase. These two 80 kDa species were shown to be distinct proteins by native gel electrophoresis and immunoblot analysis (see below), as well as by centrifugation of peak gradient fractions on a second glycerol gradient (data not shown). As shown by a variety of purification schemes, only p80 and p95 reproducibly copurified with telomerase activity (data not shown).

Active telomerase must contain protein(s) stably bound to the telomerase RNA. To examine further whether p80 and p95 were associated with the telomerase RNA, we analyzed purified fractions by a two-dimensional gel assay. A glycerol gradient sample (fraction 10 described above) was divided and loaded in each of three lanes of a native gel. After electrophoresis, one lane was analyzed for telomerase RNA by Northern blotting (Figure 2A); a second lane was layered on top of and run into an SDS–polyacrylamide gel to analyze proteins (Figure 2B); and a third lane was transferred to nitrocellulose, divided into segments, and assayed for telomerase activity (Figure 2C). In this native gel, telomerase RNA migrated to a position approximately one third to one half of the gel length (Figure 2A). Both p80 and p95 migrated in the native gel to a position similar to that of the telomerase RNA and were resolved from each other and from other proteins by denaturing gel electrophoresis (Figure 2B). Finally, telomerase activity was detected in the gel segment that corresponded to the position of the telomerase RNA, p80, and p95 in the native gel (Figure 2C). If different preparations of telomerase were similarly assayed, p80 and p95 were always present and could be clearly resolved from other proteins, including another 80 kDa polypeptide (Figure 2D).

The stoichiometry of p80:p95:telomerase RNA was estimated to be 1:1:1, on the basis of Coomassie staining of proteins relative to markers of known concentration and Northern blot analysis of the RNA relative to T7-transcribed standards (data not shown). This stoichiometry would suggest a molecular mass of 80 kDa plus 95 kDa plus 54 kDa, or approximately 230 kDa, which agrees with the molecular mass of the complex estimated by analysis of gel filtration and glycerol gradient sedimentation (200–270 kDa; see Experimental Procedures). The gel filtration and velocity sedimentation properties of telomerase were unchanged throughout the purification, suggesting that no components of the complex were lost.

Cross-Linking of Telomeric Primer to p95

To determine whether any individual proteins in telomerase preparations demonstrated specific binding to a telomeric primer DNA or to the telomerase RNA, we performed cross-linking with ^{32}P-labeled, iodouracil-substituted DNA or RNA. Iodouracil substitution permits cross-linking at relatively long wavelengths (312 versus 260 nm), reducing nonspecific excitation of unsubstituted groups and protein denaturation due to photodamage (Willis et al., 1993). Primers substituted with iodouracil were efficiently elongated by telomerase, and T7-transcribed, iodouracil-substituted telomerase RNA added back to telomerase fractions after digestion of endogenous RNA with micrococcal nuclease (MNase) efficiently restored telomerase activity to the fraction (Autexier and Greider, 1994; data not shown). When the radiolabeled, iodouracil-substituted primer $d(GT_2G_3)_2(G^IU_2G_3)$ was added to fractions and irra-

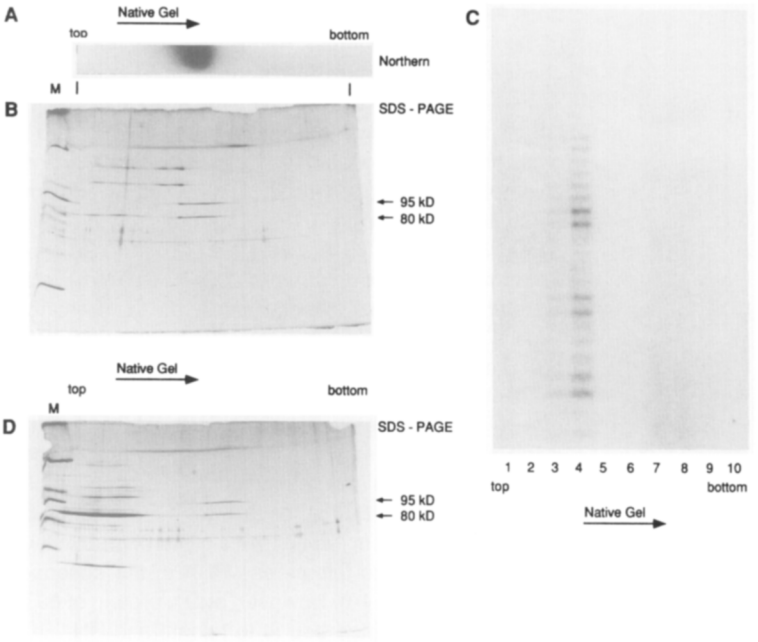

Figure 2. An Active Complex of Telomerase RNA, p95, and p80

Glycerol gradient–purified telomerase was subject to electrophoresis in three lanes of a native polyacrylamide gel. One lane was analyzed for telomerase RNA by Northern blot (A), one lane was layered on a denaturing gel and analyzed for proteins by SDS–PAGE and silver staining (B), and the third lane was transferred to nitrocellulose in immunoblot buffer, divided into segments, and assayed for telomerase activity (C). A sample from a different preparation of telomerase was also analyzed by the same techniques; the SDS–PAGE gel is shown in (D). The positions of the top and bottom edges of the native gel slices are indicated, as is the direction of native gel electrophoresis. For (C), the top of the native gel was assayed in lane 1, and lanes 3–4 corresponded to the region of the native gel at one-third the distance from the top of the gel slice. Molecular mass markers for SDS–PAGE (M) were loaded adjacent to the native gel slice in (B) and (D). The migration of p95 and p80 is indicated at the right of the gels (marked 95 kDa and 80 kDa).

diated with 312 nm light, a 95 kDa protein cross-link was observed (Figure 3). This radiolabeling persisted even after treatment of the fractions with MNase, which degraded unbound substrate DNA. This cross-linking assay differs from a previously published method (Harrington et al., 1995) in that primer elongation is not required for cross-linking.

For comparison of cross-linking to telomerase activity, fractions from the glycerol gradient shown in Figure 1 were cross-linked (Figures 3A and 3B). Cross-linking across the gradient revealed a radiolabeled protein at 95 kDa (Figure 3A) that cofractionated with activity and paralleled the presence and migration of p95 in the gel (Figure 3B). The cross-linking of proteins larger than 95 kDa was unique to this preparation of telomerase. In this preparation and others, only the 95 kDa cross-link copurified with telomerase activity.

To determine whether radiolabeling of the 95 kDa protein was specific for telomeric primers, we examined the ability of unlabeled primers to compete for binding and cross-linking of the radiolabeled substrate. In Figure 3C, cross-linking was performed in the presence of titrations of the unlabeled, 18 nt iodouracil-substituted primer (lanes 1–5); a nonspecific oligonucleotide derived from plasmid sequence (pBR; lanes 7–11); and an 18 nt unsubstituted telomeric primer (lanes 12–16). Both substituted and unsubstituted telomeric primers competed for the cross-link at concentrations equal to that of the radiolabeled primer itself (Figure 3C, lanes 3 and 14); however, even a 10-fold excess of pBR did not effectively compete for the 95 kDa cross-link (lane 11). The 95 kDa cross-link was not competed by a 60-fold concentration excess of either linear double-stranded DNA, 4.5S and 5S RNAs, or 16S and 28S RNAs (data not shown).

We also examined the specificity of the 95 kDa cross-link, relative to other protein cross-links in some gradient fractions, for competition by additional primers (Figure 3D). Telomerase more efficiently elongates 18 nt (triple-repeat) telomeric primers than 12 nt (double-repeat) telomeric primers (Collins and Greider, 1993; Lee and Blackburn, 1993). A double-repeat primer competed for the 95 kDa cross-link (Figure 3D, lanes 3–5 and 14), but less effectively than a triple-repeat primer (Figure 3C, lanes 12–16). A titration of the nonspecific pBR oligonucleotide more efficiently competed for radiolabeling of lower molecular mass proteins than the 95 kDa cross-link (Figure 3D, lanes 6–8). An oligonucleotide complementary to the telomerase RNA 3′ of the template sequence (oligo 8) can be elongated but did not compete for elongation of a telomeric primer (Greider and Blackburn, 1989). This oligonucleotide did not compete for the 95 kDa cross-link (Figure 3D, lanes 9–11). The 95 kDa cross-link was not inhibited by pretreatment of telomerase with RNase (Figure 3D, lane 12) or MNase (data not shown) under conditions that completely eliminated telomerase activity. These results suggest that primer binding by telomerase p95 did not require the presence of a functional RNA template (see Discussion).

Binding of Telomerase RNA to p80

To determine whether binding of telomerase RNA to p80 or p95 could be detected in an assay similar to that used for the primer binding described above, we synthesized ^{32}P-labeled, partially iodouracil-substituted telomerase RNA in vitro (see Experimental Procedures). Cross-linking was performed with the radiolabeled RNA, either with endogenous RNA present or after removal of endogenous RNA and reconstitution of active telomerase with the synthetic, radiolabeled RNA. An RNA cross-link was observed for an 80 kDa protein following reconstitution or if EDTA was added to telomerase without prior digestion of endoge-

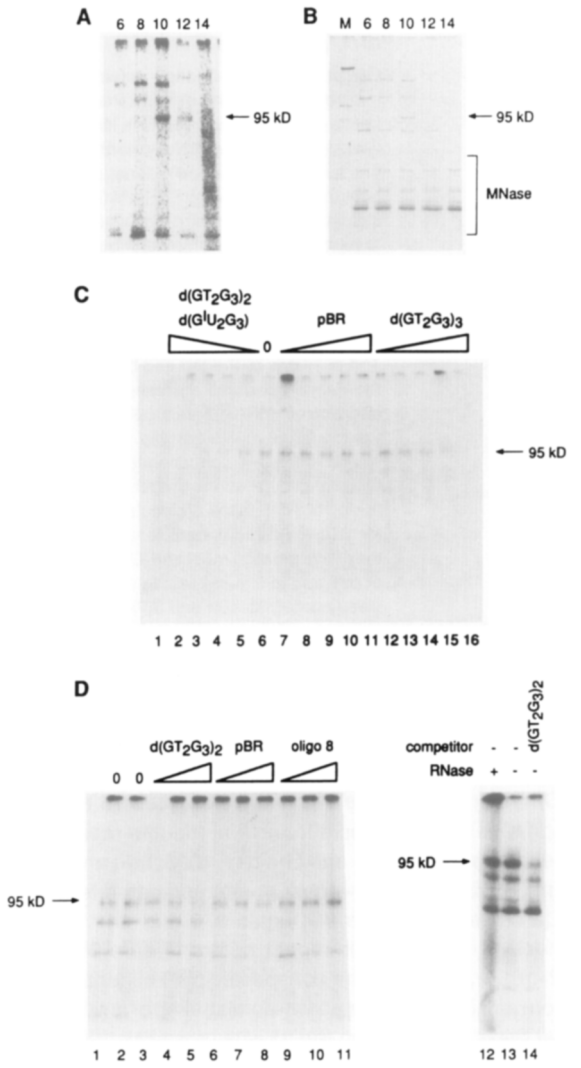

Figure 3. Cross-Linking of p95 to Primer DNA

(A and B) Autoradiograph (A) and corresponding silver-stained SDS polyacrylamide gel (B) of primer cross-linking across the indicated glycerol gradient fractions. The positions of the 95 kDa cross-link and protein are indicated. The bracket labeled MNase indicates a set of proteins common to all lanes derived from the addition of MNase after cross-linking. The M lane contains molecular mass markers, most prominently of 200, 116, and 97 kDa. Cross-linking was done in the presence of 1.5 μM unlabeled competitor oligonucleotide (pBR; see Experimental Procedures).

(C) Cross-linking competition with unlabeled oligonucleotides. The iodouracil-substituted primer used for radiolabeling (lanes 1–5), the nonspecific oligonucleotide pBR (lanes 7–11), or the unsubstituted telomeric primer of the same permutation as used for cross-linking ($d(GT_2G_3)_3$; lanes 12–16) was added to telomerase immediately before addition of radiolabeled primer. The sample in lane 6 had no competitor added (0). The concentration of competing oligonucleotide increased as indicated by the wedges, with 0.05, 0.15, 0.5, 1.5, or 5 μM final concentration compared with 0.5 μM radiolabeled primer. The position of the 95 kDa cross-link is indicated to the right, determined from silver staining of markers.

(D) Cross-linking competition with additional unlabeled oligonucleotides and after pretreatment of telomerase with RNase. Lanes 1–11 and 12–14 were from two separate gels. The oligonucleotides $d(GT_2G_3)_2$ (lanes 3–5 and 14), pBR (lanes 6–8), and oligo 8 (lanes 9–11) were added to telomerase immediately before addition of radiolabeled primer; reactions in lanes 1, 2, 12, and 13 had no competitor added. The concentration of competing oligonucleotide increased as indicated by the wedges, with 1, 3, and 10 μM final concentration compared with 0.5 μM radiolabeled primer; the sample in lane 14 contained 10 μM competitor. Prior to addition of cross-linking substrate, extract was treated with (lane 12) or without (lane 13) DNase-free RNase for 13 min at room temperature; this treatment completely destroyed telomerase activity. The position of the 95 kDa cross-link is indicated to the left of each gel, determined from silver staining of markers.

nous RNA (EDTA treatment is one step in reconstitution of telomerase and other RNPs and may facilitate exchange of endogenous RNA; Autexier and Greider, 1994). A weak cross-link was also observed at 95 kDa under the latter conditions (data not shown). Both the 80 and 95 kDa cross-links cofractionated with telomerase activity across glycerol gradients and were not competed by addition of unlabeled, nonspecific RNAs (data not shown). Unfortunately, digestion of RNA with nuclease before SDS–polyacrylamide gel electrophoresis (SDS–PAGE) removed the radiolabel from cross-linked proteins, and the presence of excess radiolabeled telomerase RNA during SDS–PAGE obscured cross-linking signals below approximately 60 kDa.

To overcome this problem, we used a different approach. Proteins were resolved by SDS–PAGE, renatured in the gel with 10% ethanol, and incubated with ^{32}P-labeled telomerase RNA in the presence or absence of unlabeled competitor RNAs. By use of this gel overlay technique, several proteins from fractions of a glycerol gradient were observed to bind to telomerase RNA in the absence of competitor (Figure 4A). However, only a protein of 80 kDa in fraction 8 remained bound to telomerase RNA in the presence of competitor RNA and cofractionated with the peak of activity in this gradient (activity not shown; Figure 4B). In some assays, a radiolabeled 95 kDa protein that cofractionated with activity was also observed (data not shown). However, the 95 kDa protein was much less intensely radiolabeled than the 80 kDa protein.

Immunoblot and Immunoprecipitation Analyses of p80 and p95

Large-scale purifications of telomerase were pooled and used to obtain internal peptide sequence from protease-digested p80 and p95 (see Experimental Procedures). Synthetic peptides were designed to match sequences derived from both subunits and were used for production and affinity purification of polyclonal antisera. With affinity-purified antibodies against p80 and p95, the distributions of p80 and p95 were examined as a function of telomerase purification. Across each chromatography step, the distributions of p80 and p95 paralleled the distribution of telomerase activity (data not shown). For the glycerol gradient assayed in Figure 1, we analyzed total protein (Figure 5A) in comparison with an immunoblot for p80 and p95 (Figure 5B). Both p80 and p95 cosedimented with telomerase activity, which peaked in fraction 10. Note that the other 80 kDa protein(s) in inactive gradient fractions was not recognized by the p80 antibody.

To determine whether p80 and p95 are integral components of telomerase, we tested the ability of the affinity-

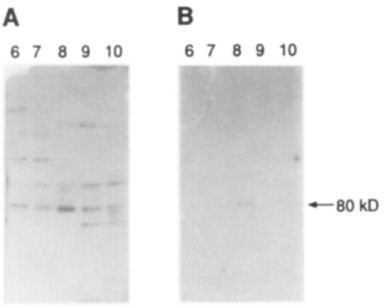

Figure 4. Binding of p80 to the Telomerase RNA

The indicated fractions from a glycerol gradient were resolved in duplicate on one SDS–polyacrylamide gel. Half of the gel was incubated with radiolabeled telomerase RNA alone (A), and half of the gel was incubated with radiolabeled telomerase RNA and competitor 4.5S and 5S RNA (B). Arrows indicate the migration of p80, determined from prestained molecular mass markers on the gel.

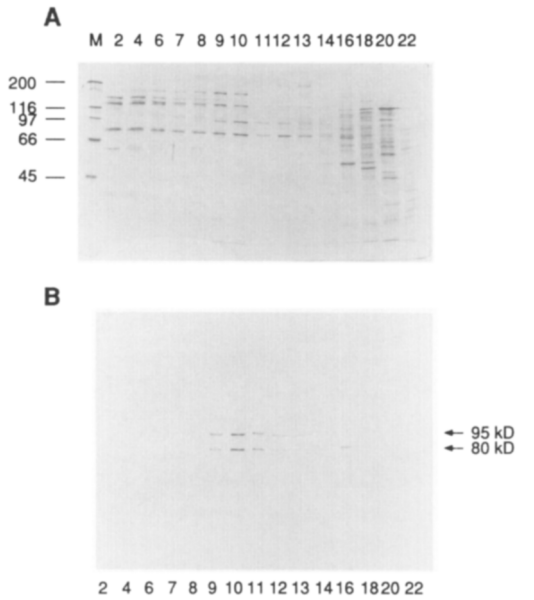

Figure 5. Immunoblot Analysis of Telomerase Purification

The indicated fractions from the glycerol gradient in Figure 1 were run in parallel on two gels. Telomerase activity was predominantly observed in fractions 9–12 and peaked in fraction 10. One gel was silver stained (A), while the other was transferred to nitrocellulose and probed with combined p80 and p95 antibodies (B). The M lane in (A) contains molecular mass markers as indicated. Arrows indicate the migration of p95 and p80, determined from prestained molecular mass markers on the gel.

purified antibodies to immunoprecipitate telomerase activity. Antibodies were bound to protein G–Sepharose beads, and then antibody-coated beads were incubated with partially purified telomerase, pelleted, and washed. Supernatant, wash, and bead fractions were assayed for telomerase activity. Two kinds of control immunoprecipitations were performed. First, we used an affinity-purified anti-peptide antibody not specific for telomerase. Second, specific antibody was used in the presence of competitor peptide. The p80 antibody specifically immunoprecipitated telomerase activity, even from extracts of greater than 0.4 M ionic strength (Figure 6A). Telomerase activity was present in the bead fraction of immunoprecipitations with p80 antibody (Figure 6A, lane 6), but was greatly reduced in precipitates with p80 antibody and competitor peptide (lane 9) or in precipitates with the nonspecific antibody (lane 3). At reduced ionic strength, immunoprecipitation by p80 antibody was greatly enhanced, but some telomerase was recovered nonspecifically in the bead fraction of a control immunoprecipitation (data not shown). The p95 antibody did not immunoprecipitate active telomerase. At low ionic strength, telomerase activity was depleted from the supernatant of p95 immunoprecipitations (data not shown). These results suggest that binding of p95 to the antibody-coated beads inhibited telomerase activity.

We examined the bead fraction of p80 immunoprecipitations for the presence of p95. Immunoblotting revealed that p95 was present in the bead fraction containing active telomerase (Figure 6B, lane 2). The amount of p95 precipitated was reduced in the presence of competitor peptide or in precipitations with nonspecific antibody (Figure 6B, lanes 1 and 3). Immunoprecipitation samples were also assayed for the presence of telomerase RNA. Telomerase RNA was depleted from the supernatant and recovered in the bead fraction of p80 and p95 immunoprecipitations (data not shown). However, owing to incomplete recovery of RNA from bead fractions, we could not reliably quantitate immunoprecipitation of telomerase by Northern blot. We conclude that immunoprecipitation by p80 antibody recruited both telomerase RNA and p95 to the bead fraction containing active telomerase.

Isolation of Genes Encoding p80 and p95

Peptide sequence from p80 and p95 was used to design degenerate oligonucleotides, which were used in various combinations for either reverse transcription and polymerase chain reaction (PCR) from Tetrahymena poly(A)$^+$ RNA or for PCR from macronuclear genomic DNA. PCR products were cloned and sequenced; if the DNA fragments encoded peptide sequence obtained from direct sequencing but not included in the primers, they were judged to be correct. Two correct PCR products were obtained for each gene (see Figure 7A). These PCR products were used to screen a Tetrahymena cDNA library, but only partial clones were obtained (see Figure 7A). Next, genomic libraries were constructed with EcoRI- or ClaI-digested DNA and screened with the available probes. Only one EcoRI fragment including a portion of the coding region was recovered for each gene (Figure 7A). Genomic Southern blots with the PCR products demonstrated the presence of additional genomic restriction fragments that were unstable for propagation in Escherichia coli and revealed that both genes were single copy in the macronuclear genome (data not shown). Protocols utilizing rapid amplification of cDNA ends (RACE) were used to obtain the 5′ end of the p80 cDNA and 5′ and 3′ ends of the p95 cDNA; the 3′ end of the p80 cDNA was also mapped by this technique (Figure 7A). All sequence obtained from protocols, includ-

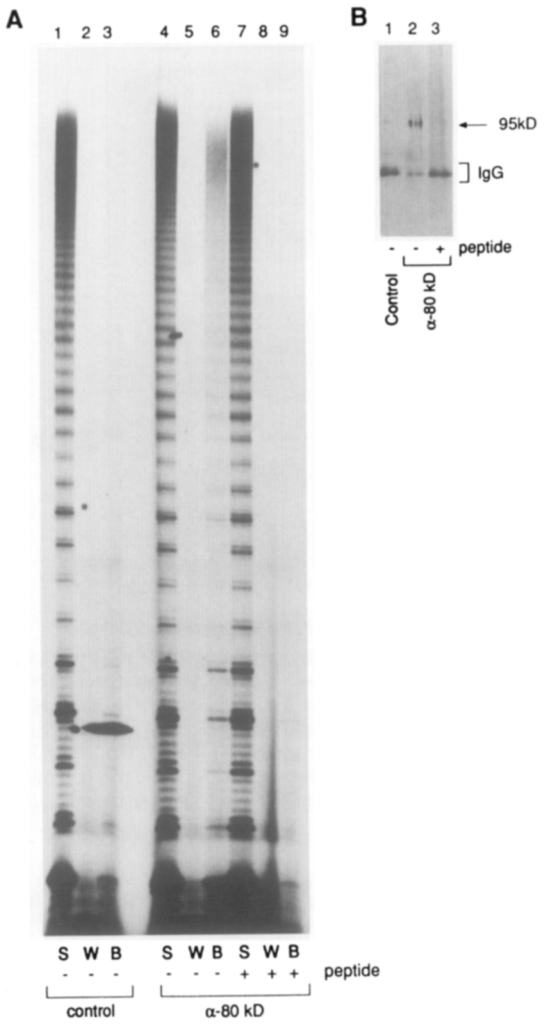

Figure 6. Immunoprecipitation of Telomerase

(A) Telomerase activity assayed in supernatant (S), third wash (W), and bead (B) fractions of immunoprecipitations. Either control (lanes 1–3) or p80-specific (lanes 4–9) antibody was used, in the absence (lanes 1–6) or presence (lanes 7–9) of competitor p80-derived peptide.

(B) Immunoblot for p95 with precipitated bead fractions. Lane 1 was from control antibody precipitation, lane 2 was from p80 antibody precipitation in the absence of peptide, and lane 3 was from p80 antibody precipitation in the presence of competitor peptide. The p80 antibody bound to protein G before immunoprecipitation was present in the bead fraction and was recognized by the secondary antibody, indicated to the right of the gels as IgG. Protein standards were included on the gel and were transferred to nitrocellulose to verify the migration of the cross-reacting polypeptides.

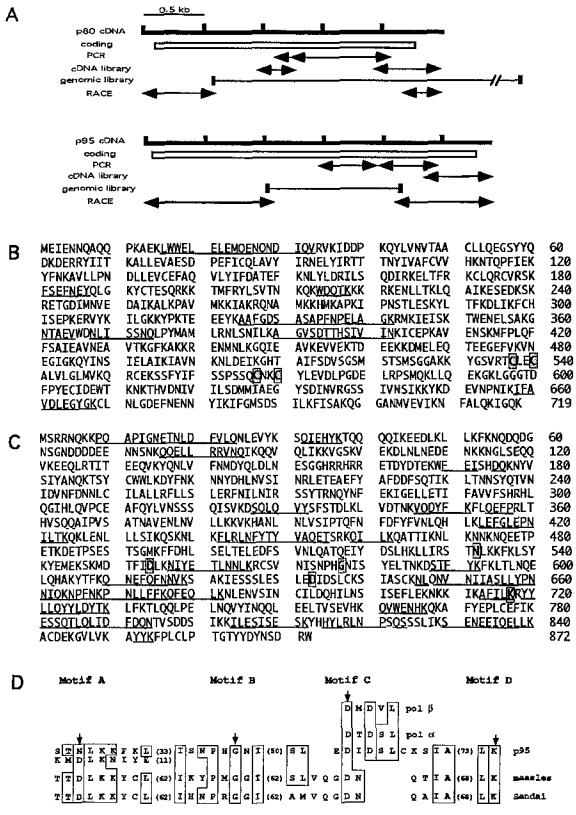

Figure 7. Isolation and Analysis of Telomerase Protein Genes

(A) Gene fragments obtained from various cloning strategies. Regions isolated by PCR, from a cDNA library, from an EcoRI genomic library, or from RACE protocols are indicated in their positions along the full-length cDNAs. The coding regions are indicated by open boxes; PCR, λ phage, and RACE products by lines with arrowheads; and genomic fragments by lines with perpendicular ends (the p80 genomic fragment was 3.2 kb).

(B and C) Predicted amino acid sequences of p80 (B) and p95 (C). Translation of the longest open reading frame of each cDNA is shown, from the first AUG codon. Sequence unambiguously identified by direct peptide sequencing is underlined. Boxed and stippled letters denote sequence features described in the text and in (D) (putative metal-binding motif in p80 and putative polymerase motifs in p95).

(D) Putative polymerase active site motifs in p95. The sequence shown for p95 begins at amino acid 531. The p95 sequence contained two reasonable alignments to motif A, one 33 and one 11 amino acids N-terminal to the motif B sequence; both are shown. Other sequences are taken from compilations (Delarue et al., 1990; Poch et al., 1989). Arrowheads labeled motifs A–D indicate the position of the most conserved amino acid within each motif. For p95, the positions of these amino acids in the protein sequence are indicated in (C) by stippled boxes.

ing reverse transcription (cDNA library clones and RACE products), was verified by sequencing PCR products amplified from genomic DNA. No difference was found in cDNA and genomic sequences, indicating that neither gene is disrupted by introns. The complete sequence of cDNAs for p80 and p95 (and derived amino acid sequences) have been deposited in GenBank.

Translations of the longest open reading frames of the p80 and p95 cDNAs are shown in Figures 7B and 7C. In Tetrahymena, the codons UAA and UAG specify glutamine; the only nonsense codon is UGA (for review, see Prescott, 1994). The coding regions cloned are likely to be complete on the basis of several criteria. First, Northern blot analysis of the p80 and p95 mRNAs suggests sizes of approximately 2.5 and 2.9 kb (data not shown); we have obtained greater than 2.4 and 2.8 kb of sequence for these mRNAs. Second, all peptide sequences obtained directly were found in these open reading frames (7 of 7 peptides for p80; 25 of 25 peptides for p95). Third, translation of the mRNAs from the first AUG codon in the longest open reading frame yielded predicted protein products of equal

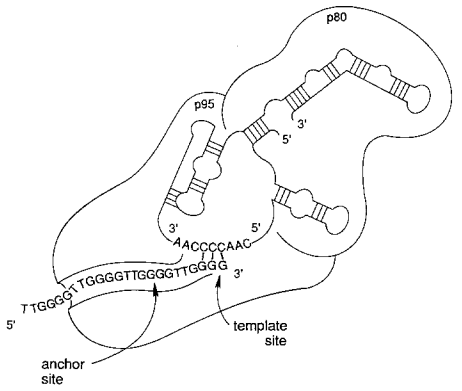

Figure 8. Working Model for the Subunit Structure of the Tetrahymena Telomerase RNP

Telomerase RNA secondary structure is drawn largely as proposed (Romero and Blackburn, 1991; Bhattacharyya and Blackburn, 1994). Telomeric primer $d(T_2G_4)_4$ is shown in a state of elongation. Base pairing promotes binding of the 3′ end of a primer at the template site. The p95 subunit contains some or all of a template-independent primer-binding site termed the anchor site (Collins and Greider, 1993). The p80 subunit binds tightly and specifically to the telomerase RNA, suggesting its association with a region of RNA secondary structure.

or slightly greater molecular mass than that estimated from SDS–PAGE analysis. Fourth, sequences outside the translated coding regions are enriched in runs of A and T, typical of Tetrahymena genes; the untranslated regions also have lengths typical of Tetrahymena genes (Prescott, 1994).

Comparisons of p80 and p95 with available database sequences revealed that the telomerase proteins are not highly related to each other or to other known sequences, including Est1, a putative yeast telomerase component (Lundblad and Szostak, 1989). Alignments of polymerase sequences have defined four regions, termed motifs A–D, that characterize a polymerase active site (Delarue et al., 1990; Poch et al., 1989). Although the conservation of these motifs across all polymerases is restricted to one or a few amino acids per motif, families of related polymerases share additional conserved residues. Searches of the databases using FASTA or BLAST (Altschul et al., 1990) with limited regions of p80 and p95 sequence, as well as direct comparison of telomerase and other polymerase sequences, yielded an alignment of p95 with the polymerase active site of a family of viral RNA-dependent RNA polymerases. Many residues conserved between the viral RNA-dependent RNA polymerases, represented in Figure 7D by measles and Sendai, are also conserved in p95. These include the isoleucines and prolines of motif B. The suggested p95 motif C is more related to motif C from α- and β-like polymerases than to motif C from the RNA-dependent RNA polymerases in the spacing of aspartic acids by a hydrophobic residue (Figure 7D). In isolation, other sequences of the p80 and p95 subunits could also be aligned as a motif C. Future mutagenesis studies will address whether the region of p95 indicated in Figure 7D is important in the catalysis of the polymerization reaction.

In addition to the homology described above, we noted the presence of several other sequence features. A potential metal-binding motif is present in p80, with the structure CxxC(27)CxxC (residues 537–570). The spacing of the cysteines in this motif resembles that of the zinc-binding motifs of transcription factors, including TFIIS and TFIIE (Hirashima et al., 1988; Peterson et al., 1991; Ohkuma et al., 1991), as well as other proteins. Other potential RNA and nucleotide-binding motifs found in p95 include several arginine dipeptides and a class II aminoacyl-tRNA synthetase motif 3 (residues 258–267; see Discussion).

Discussion

Subunit Structure and Function in the Telomerase RNP

This report describes the purification and characterization of telomerase protein components. Our results suggest that Tetrahymena telomerase is composed of two protein subunits, p80 and p95, in a 1:1:1 stoichiometry with each other and the 159 nt telomerase RNA (Figure 2). Antibodies raised against p80 specifically immunoprecipitated telomerase activity, telomerase RNA, and p95, demonstrating that p80 and p95 are integral components of telomerase (Figure 6). After treatment of telomerase with RNase or MNase, p80 and p95 remained associated, whether analyzed by glycerol gradient sedimentation or native gel electrophoresis (data not shown). Thus, protein–protein as well as protein–RNA interactions occur in the telomerase RNP.

The two protein subunits of Tetrahymena telomerase have unique nucleic acid binding properties. The specific binding of p80 to the telomerase RNA (Figure 4) suggests that this subunit might recognize an element of RNA secondary structure, such as a conserved stem–loop region, that would not be present in the competitor ribosomal RNAs. The binding of DNA to p95, analyzed by our cross-linking assay (Figure 3), reflects the specificity expected for primer binding at the anchor site: longer telomeric primers competed for the cross-link at lower concentration than shorter primers, and telomeric primers competed at lower concentrations than nonspecific oligonucleotides. Cross-linking was not inhibited by pretreatment of telomerase with RNase, indicating that primer binding does not require a functional template sequence or the integrity of the telomerase RNA.

These observations suggest a division of labor in the telomerase RNP (Figure 8). If p80 binds to telomerase RNA secondary structure, p95 binds to primer at the anchor site, and if p80 and p95 interact with each other, then the template sequence of the RNA, the 3′ end of a primer DNA, and the active site could all be brought together in a flexible conformation. This arrangement would allow all components to form a stable RNP, yet leave the template and elongating product constrained only by synthesis at the active site. A low affinity binding of p95 to telomerase RNA was observed in some cross-linking and overlay assays; perhaps this reflects the association of p95 with RNA regions that must remain flexibly tethered in the ac-

tive RNP. Movement of the active site or of the RNA template relative to the rest of the telomerase RNA and p80 could occur during synthesis of one 6 nt repeat. This relative displacement would be reset when the product 3′ end realigned with the 3′ end of the template (the so-called translocation event). Alternatively, movement of product through the anchor site, in increments of either single nucleotides or 6 nt repeats, could provide a mechanism for resetting the product 3′ end at the 3′ end of the template.

Primary Structure of the Tetrahymena Telomerase Proteins

Owing to the lack of extensive sequence homology between telomerase proteins and other polymerases, telomerase appears to represent a novel type of polymerase. However, biochemical data suggest that the limited sequence similarity that we detect between telomerase and viral RNA-dependent RNA polymerases could have a functional basis. Telomerase binds and utilizes an RNA template. Telomerase also binds and elongates primers of RNA and incorporates ribonucleotides (K. C. and C. W. G., submitted). This capacity of telomerase for elongating and synthesizing RNA may have an unknown significance for its function in vivo or may reflect a common branch of evolutionary history for telomerase and viral RNA-dependent RNA polymerases. Also interesting in light of evolutionary proposals for early functions of tRNA-like structures (Maizels and Weiner, 1993) is the presence of a class II tRNA synthetase signature motif 3 in p95. This motif, with the aid of a magnesium ion coordinated by residues outside the motif, serves to position a reactive nucleotide triphosphate at the synthetase active site (Cavarelli et al., 1994).

The p80 subunit contains a putative zinc-binding motif that is similar in the spacing of cysteines to the zinc-binding motifs of eukaryotic RNA polymerase transcription factors TFIIE and TFIIS. TFIIE functions during transcription initiation, promoting the forward movement of RNA polymerase from a stable open promoter complex (Goodrich and Tjian, 1994). TFIIS functions during transcription elongation, also promoting movement of a stalled polymerase by stimulating the nucleolytic cleavage of product RNA (for review, see Kassavetis and Geiduschek, 1993). The nucleolytic cleavage reaction of RNA polymerases has many similarities to the nucleolytic cleavage reaction catalyzed by telomerase (Collins and Greider, 1993). Thus, the CxxC(n)CxxC motif of p80 may have functional as well as structural homology with the similar motif present in the RNA polymerase transcription factors described above. Perhaps stalled telomerase and RNA polymerase enzymes share the requirement for disruption of an overly stable DNA–RNA duplex. The common CxxC(n)CxxC motif could function in binding this duplex or in binding single-stranded template or product near the active site.

Experimental Procedures

Purification of Telomerase

Tetrahymena thermophila were grown at 30°C to a density of 4 × 10^5/ml in PPYS (2% proteose peptone, 0.2% yeast extract, 10 μM $FeCl_3$), harvested by centrifugation in a GSA rotor (Sorvall), and starved for 18 hr in Dryl's solution (1.7 mM $NaC_6H_8O_7$, 1.2 mM NaH_2PO_4, 1.3 mM Na_2HPO_4, 2 mM $CaCl_2$). Starved cells were again harvested, resuspended in T2MG buffer (20 mM Tris–HCl [pH 8.0], 1 mM $MgCl_2$, 10% glycerol, 2 mM DTT or β-mercaptoethanol, 0.1 mM PMSF, 2 μg/ml leupeptin, 1 μg/ml pepstatin), and lysed by addition of a final concentration of 0.2% NP-40. S-130 extract was obtained by centrifugation of lysed cells in a Ti45 rotor (Beckman) at 40,000 rpm for 50 min at 4°C. All subsequent steps were done at 4°C.

S-130 extract was filtered coarsely and applied to ceramic HAP (American International Chemicals, Incorporated) equilibrated in T2MG. Telomerase was eluted with a gradient to 0.2 M K_2HPO_4 in T2MG. Fractions with peak activity were pooled, diluted with 3 vol of T2MG, and applied to spermine–agarose (Sigma) equilibrated in T2MG with 0.15 M potassium glutamate (abbreviated KG). Telomerase was eluted in T2MG with 0.65 M KG. Fractions with peak activity were pooled and loaded on a 1 liter column of Sepharose CL-6B (Pharmacia), equilibrated, and run in T2MG with 20 mM KG and 3 mM NaN_3. Fractions with peak activity were pooled, adjusted to 0.4 M KG, and applied to phenyl–Sepharose (Pharmacia) equilibrated in T2MG with 0.4 M KG. The column was washed in T2MG, and then telomerase was eluted in T2MG with 1% Triton X-100. Fractions with peak activity were pooled and applied to DEAE–agarose (Bio-Rad) or Q-Sepharose (Pharmacia) equilibrated in T2MG. Telomerase was eluted with a step to 0.4 M KG or a gradient to 2.0 M KG in T2MG. Fractions with peak activity were diluted, usually with distilled water, layered on 15%–35% or 20%–35% glycerol gradients in 20 mM Tris–acetate, 1 mM $MgCl_2$, and centrifuged for 20 hr in an SW41 rotor (Beckman). The maximum recovery of telomerase from a large-scale (300 liter) Tetrahymena prep was obtained from an S-130 extract (19 g of protein, 30 μg of telomerase RNA) at a 47,619-fold purification of telomerase RNA relative to total protein after glycerol gradient centrifugation. After native gel electrophoresis, a total of 10 μg of telomerase proteins was recovered for a total of 191,191-fold purification.

By glycerol gradient sedimentation, telomerase fractionated with an apparent molecular mass of approximately 270 kDa. FPLC chromatography of telomerase or standards on a Superose 6 column suggested a molecular mass of 200 kDa for the telomerase complex.

Telomerase Activity

For analysis of product DNA on denaturing gels, telomerase activity was assayed as described (Greider and Blackburn, 1989). During purification, telomerase activity was assayed as above for 20 min and stopped by addition of EDTA, and a fraction of the reaction was slot-blotted to DE81 paper by vacuum. The DE81 paper was washed in 0.5 M Na_2HPO_4 three times for 10–15 min each, rinsed in water and 95% ethanol, and dried. Activity was quantitated by a phosphorimager (Fuji).

Native Gels

Fractions were adjusted to at least 10% glycerol and loaded on a 6% polyacrylamide, 50 mM Tris–acetate minigel. The native gel was run in 50 mM Tris–acetate buffer (pH 8.0) at 150 V (minigel) or 250 V (preparative gel) for up to approximately 12 hr. Gel lanes were excised, soaked briefly in SDS sample buffer, and sealed into the well of a denaturing gel with 0.1% agarose in 25 mM Tris–acetate.

Cross-Linking

Iodouracil-substituted DNA was synthesized with iodouracil phosphoramidite (Peninsula Labs), end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Sambrook et al., 1989), and desalted. Samples for cross-linking were adjusted to 1 × telomerase reaction buffer and 0.5 μM radiolabeled primer, equilibrated at 30°C for 10 min, and cross-linked in a Stratalinker equipped with 312 nm bulbs. Irradiation with 500 mJ was used, as it was maximal within the linear range of increase in cross-link intensity. After cross-linking, unbound primer was digested with 25 U of MNase in 1 mM $CaCl_2$ for 15 min at room temperature. Samples were adjusted with sample buffer and subjected to SDS–PAGE. Gels were silver stained and dried. Oligonucleotide sequences are as follows: pBR, dAGCCACTATCGACTACGCGATCAT; oligo 8, dGAAGGTTATATCAGCACTAGATTT.

RNA Binding Assays

Iodouracil-substituted, ^{32}P-labeled telomerase RNA was made by T7

transcription using the conditions described (Autexier and Greider, 1994), with the following changes. All four unlabeled rNTPs were added at 500 μM, [α-^{32}P]rATP was added at 150 nM, and iodouracil triphosphate (Sigma) was added at 500 μM. After DNase treatment of the reaction, RNA was gel-purified or purified by RNaid resin (BIO 101).

For gel overlays, SDS gels were incubated twice for 10–15 min in 50% methanol and for at least 4 hr in 10% ethanol to renature proteins partially. Gels were then equilibrated in overlay buffer (50 mM Tris–acetate, 10 mM $MgCl_2$, 10% glycerol). Radiolabeled telomerase RNA (10 μg) was heated with or without a mixture of 4.5S and 5S RNA, sold as 5S RNA (Boehringer Mannheim), and added to gels in 10 ml of overlay buffer. After overnight incubation, washes were done with overlay buffer.

Peptide Sequencing

Fractions from multiple glycerol gradients were pooled and applied to DEAE–agarose equilibrated in T2MG. Telomerase was eluted in T2MG with 0.4 M KG. Peak fractions were dialyzed against T2MG and then fractionated by native and denaturing gel electrophoresis. SDS gels were stained with Coomassie brilliant blue (Aldrich). Polypeptides were excised from the gel after soaking 10 min in distilled water. Gel slices were minced and soaked in 50% methanol twice for 20 min, decanted, and dried briefly under vacuum. Proteins were digested with approximately 300 ng of Achromobacter protease I in 0.1 M Tris–HCl (pH 9.0), 0.01% Tween-20 for 24 hr at 30°C. Peptides were separated from gel fragments by spin filtration, concentrated by Speed-Vac, and applied to a Vydac C-18 column (2.1 mm × 250 mm). Peptides were eluted with a gradient of acetonitrile:isopropanol (3:1) in 0.09% trifluoroacetic acid. Peaks of absorbance at 214 nm were collected, lyophilized, and applied to a protein sequencer (Appied Biosystems).

Antibodies, Immunoblots, and Immunoprecipitation

Peptides with N-terminal cysteines were synthesized (Genosys) from sequences of p80 (CAEGTSDINVRG) and p95 (CEFGLEPNILK; the p95 peptide skips one threonine residue of the native protein), conjugated via Sulfo-SMCC (Pierce catalog number 22322) to keyhole limpet hemocyanine (Sigma), and used to immunize rabbits (Hazelton). For affinity purification, sera were mixed 1:1 with PBS and loaded onto columns covalently modified with the peptide (Pierce catalog number 44895). Columns were washed in PBS plus 0.5 M NaCl and eluted sequentially with 0.1 M sodium acetate (pH 4.0) and 0.1 M glycine (pH 2.7).

For immunoblots, gels were equilibrated in 1 × buffer (10 mM CAPS [pH 11], 20% methanol) and then transferred to nitrocellulose at 0.5 M for 1 hr. Nitrocellulose was blocked in PBS with 5% BSA, incubated with affinity-purified antibody in blocking buffer, washed with PBS plus 0.05% NP-40, incubated with alkaline phosphatase–conjugated goat anti-rabbit antisera (Sigma), and washed as above. Blots were developed with NBT and BCIP (Sigma).

Affinity-purified antibodies diluted in PBS were bound to protein G–Sepharose (Pharmacia). Beads were washed twice with 20 vol of PBS and twice with 20 vol of binding buffer (T2MG with 0.15 [Figure 6B] or 0.4 M [Figure 6A] KG and 0.05% NP-40). Telomerase fractions were diluted with several volumes of binding buffer and mixed end over end with antibody-bound beads for 4 hr at 4°C. Beads were washed three times with 20 vol of binding buffer and resuspended in binding buffer at one-fifth the volume of the diluted extract used for binding. Equal volumes of the unbound supernatant, the third bead wash, and the resuspended beads were assayed for telomerase activity. In the reactions with competitor peptide, approximately 10–100 μg of peptide in distilled water was added to the antibody-bound beads immediately prior to addition of telomerase.

Gene Cloning

Degenerate primers were designed from peptide sequences with consideration of Tetrahymena codon usage frequencies (Martindale, 1989). RNA was isolated from starved Tetrahymena by the method of guanidine isothiocyanate extraction (Ausubel et al., 1992) and selected on poly(dT) cellulose (New England Biolabs) according to standard protocol (Ausubel et al., 1992). Reverse transcription of poly(A)$^+$ RNA with degenerate oligonucleotides was done using the Superscript and Superscript II derivatives of MMLV reverse transcriptase (GIBCO). Reactions were performed at 50°C according to the instructions of the manufacturer, digested with RNase H and RNase A, and precipitated. Macronuclear genomic DNA was prepared as described elsewhere (Cherry and Blackburn, 1985) and denatured before PCR by incubation at 95°C for 5 min. A variety of PCR conditions was required. Products from PCR amplification were purified, cloned in E. coli, and sequenced by standard protocol (United States Biochemicals). The sequences of primers that gave PCR products indicated in Figure 7A follow, from 5′ to 3′ of the cDNA, with (S) indicating sequence of a sense strand and (C) indicating sequence of the complementary strand. For p95, sequences are GA(A/G)TT(T/C)GG(T/A/C)(T/C)TNGA(A/G)CC (S), (A/G)TT(A/G)TT(A/G)AA(T/C)T(A/G)(A/G)AA(T/C)TC(A/G)T T (C), AA(T/C)GA(A/G)TT(T/C)(T/C)A(A/G)TT(T/C)AA(T/C)AA (S), and (C/T)TT(A/G)TT(T/C)T(A/G)(A/G)TC(A/G)AA(A/G)TC(A/T/G)AT (C). For p80, sequences are GC(T/C)CC(T/C)TT(T/C)AA(T/C)CC(T/C)GA (S), GC(T/C)GG(T/A)GT(T/C)(T/A)(C/G)(T/C/A)GA(T/C)AC(T/C) AC (S), and (T/C)TT(T/A)CC(A/G)TA(T/A)CC(T/A)TCNA(A/G)(A/G)TC (C).

Products obtained by PCR were hexamer labeled (Sambrook et al., 1989) and used to screen an oligo(dT)-primed Tetrahymena cDNA library in λgt10 (Takemasa et al., 1989). Hybridization was performed in buffer of 6 × SSC, 0.1% SDS, 5 × Denhardt's solution, and 50 μg/ml salmon testes DNA (Sigma) at 60°C. Washes were at the same temperature with 2.0–0.2 × SSC, 0.1% SDS. Genomic libraries were constructed in Bluescript KS(+) (Stratagene) with EcoRI- or ClaI-digested macronuclear genomic DNA. Hybridization was performed in buffer of 25 mM KPO_4 (pH 7.2), 5 × Denhardt's solution, 5 × SSC, 50% formamide, and 50 μg/ml salmon testes DNA at 42°C. Washes were at the same temperature with 1.0–0.25 × SSC, 0.1% SDS.

To obtain the 5′ end of the cDNAs for both genes, RACE was done (GIBCO catalog number 18374-025) using poly(A)$^+$ RNA from starved cells. The 3′ RACE was performed on the basis of the protocol above, using oligo(dT) primer for reverse transcription. The 3′ end of the p95 cDNA was examined by comparison of λ clone and 3′ RACE product sequences. The 3′ end of the p80 cDNA was determined by comparison of the sequences of λ and genomic clones: the p80 λ clones terminated with a poly(A) sequence present only as four adenine residues in the genomic clone. The results of 3′ RACE for p80 support this region as the site of polyadenylation.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are U25641 for p80 and U25642 for p95.