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Functional reconstitution of wild-type and mutant *Tetrahymena* telomerase

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Telomerase is a ribonucleoprotein that catalyzes telomere elongation *in vitro* and *in vivo*. The 159-nucleotide RNA component of *Tetrahymena* telomerase contains the sequence 5'-CAACCCCAA-3' ("template region"), which serves as a template for the addition of the sequence d(TTGGGG)_n to *Tetrahymena* telomeres. To dissect the *Tetrahymena* telomerase enzyme mechanism, we developed a functional *in vitro* reconstitution assay. After removal of the essential telomerase RNA by micrococcal nuclease digestion of partially purified telomerase, the addition of *in vitro*-transcribed telomerase RNA reconstituted telomerase activity. The reconstituted activity was processive and showed the same primer specificities as native telomerase. Mutants in the RNA template region were tested in reconstitution assays to determine the role of the residues in this region in primer recognition and elongation. Two template mutants, encoding the sequences 5'-UAACCCCAA-3' and 5'-UAACCCUAA-3', specified the incorporation of dATP into the sequence d(TTAGGG). Telomerase reconstituted with a template mutant encoding the sequence 5'-CAACCCUAA-3' did not specify dATP incorporation and elongation by this mutant was not terminated by the addition of ddATP. In addition, a template mutant encoding the sequence 5'-CGGCCCAA-3' specified the incorporation of ddCTP but not ddTTP while a mutant encoding the sequence 5'-CAACCCGG-3' specified the incorporation of ddTTP but not ddCTP. These data suggest that only the most 5' six residues of the template region dictate the addition of telomeric repeats.

[Key Words: Telomeres; telomerase; reconstitution; ribonucleoprotein; *Tetrahymena*; micrococcal nuclease]

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The maintenance of telomere length is vital to the integrity of chromosomes and the survival of cells. One mechanism by which telomere length is maintained in eukaryotes is by the *de novo* addition of sequences onto chromosome ends by the enzyme telomerase (for review, see Blackburn 1991). *Tetrahymena* telomerase is a ribonucleoprotein (RNP) enzyme that synthesizes the telomeric sequence d(TTGGGG)_n comprising the ends of *Tetrahymena* chromosomes (Greider and Blackburn 1987). The 159-nucleotide RNA component of *Tetrahymena* telomerase contains the sequence 5'-CAACCCCAA-3' ("template region"), which serves as an internal template for the addition of telomeric sequence repeats (Greider and Blackburn 1989). When altered telomerase RNA genes, encoding mutations in the template region of telomerase RNA, were introduced into *Tetrahymena*, altered telomere sequences were synthesized that corresponded to the mutated RNA sequences, indicating that the template region directs the synthesis of telomeric repeats (Yu et al. 1990).

In vitro, *Tetrahymena* telomerase can processively elongate telomeric substrates generating a distinct six nucleotide banding pattern (Greider and Blackburn 1985). The strongly labeled product within the repeat

pattern represents a pause in elongation after the addition of the first G residue in the sequence GGGGTT (Greider 1991a). Following this pause, which occurs at the extreme 5' end of the template region, the enzyme translocates to initiate another round of telomeric repeat synthesis (Greider and Blackburn 1989). The sequence at the 3' end of telomeric substrates specifies the first nucleotide added in elongation reactions with telomerase. For example, a G residue will first be added to a telomeric primer ending in the sequence d(GGGGTT) while a T residue will be added to a primer ending in the sequence d(TTGGGG) (Greider and Blackburn 1987; Zahler and Prescott 1988; Morin 1989; Shippen-Lentz and Blackburn 1989). *Tetrahymena* telomerase also catalyzes a specific nucleolytic activity that cleaves substrates containing the telomeric repeat d(GGGGTTG) at the 3' end (Collins and Greider 1993). Telomerase activity has also been identified in the ciliates *Euplotes* and *Oxytricha* and in immortalized human and mouse cell lines (Zahler and Prescott 1988; Morin 1989; Shippen-Lentz and Blackburn 1989; Counter et al. 1992; Prowse et al. 1993). However, only the RNA components of *Tetrahymena* and *Euplotes* telomerase have been identified to date (Greider and Blackburn 1989; Shippen-Lentz and Blackburn 1990).

The presence of short tandem repeats of simple G-rich

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sequences at the ends of chromosomes is highly conserved in eukaryotes, suggesting conserved function, and/or synthesis of telomeres (for reviews, see Zakian 1989; Blackburn 1991; Greider 1991b; Biessmann and Mason 1992). Telomeres are essential for providing stability to chromosomes. Broken chromosomes that lack telomeres are generally unstable and contribute to genome rearrangements (Müller 1938; McClintock 1941; Biessmann and Mason 1992). Telomeres also ensure the complete replication of linear chromosomes. The addition of sequences by telomerase may balance sequence loss due to the incomplete replication of chromosome ends by conventional DNA polymerases (Watson 1972; Olovnikov 1973).

In *Tetrahymena* cells expressing mutant telomerase RNAs, altered telomere sequences and lengths result in nuclear and cell division defects, and senescence (Yu et al. 1990). Yeast *est1* mutants that lose telomeric sequences with each round of replication also have a senescence phenotype (Lundblad and Szostak 1989). When primary mammalian cells are passaged in vitro, telomere length decreases and the cells senesce (Harley et al. 1990). A similar shortening in telomere length is seen in vivo; fibroblast and lymphocyte telomere lengths decrease as humans age (Allsopp et al. 1992; Vaziri et al. 1993). Telomerase has been identified in many immortalized human and mouse cell lines but not in most primary cells tested to date (Morin 1989; Counter et al. 1992; Prowse et al. 1993). These data suggest that telomerase activity may be absent in primary cells and may be required for the growth of immortalized cells (Harley et al. 1990; Hastie et al. 1990; Prowse et al. 1993). To understand the role of telomerase in senescence and immortalization it is essential to elucidate the mechanism of telomerase action in the maintenance of telomere length.

To understand telomerase function we are analyzing the mechanism and components of this unusual RNP. RNPs play important roles in vital cellular processes such as protein biosynthesis, RNA splicing and processing, and protein translocation. The reconstitution of ribosomes, ribonuclease P, signal recognition particle (SRP), small nuclear RNPs (snRNPs), heterogenous nuclear RNP and human mitochondrial RNA processing RNP (MRP) has facilitated a detailed understanding of the structure, function and assembly of these enzymes (Traub and Nomura 1969; Nierhaus and Dohme 1974; Kole and Altman 1979; Walter and Blobel 1983; McPheeters et al. 1989; Schenkel and Kloetzel 1989). To dissect *Tetrahymena* telomerase we developed a functional in vitro reconstitution assay. The addition of in vitro-transcribed telomerase RNA to micrococcal nuclease (MNase)-inactivated telomerase reconstituted activity. Analysis of telomerase activity reconstituted with telomerase RNAs mutated in the template region suggests that only the most 5' six nucleotides in the sequence 5'-CAACCCAA-3' specify nucleotide sequence addition. The ability to reconstitute telomerase activity from isolated RNA and protein components will facilitate the identification of telomerase protein subunits and the detailed functional dissection of this RNP.

Results

Reconstitution of telomerase activity after MNase digestion

The integrity of many RNPs is dependent on the presence of divalent cations. Chelating agents such as EDTA have been successfully employed to partially unfold a number of RNPs including ribosomal subunits, RNase P, small cytoplasmic RNPs and SRP, which were subsequently disassembled into protein and RNA components by column chromatography (Blobel 1971; Spirin 1974; Newton et al. 1975; Guthrie and Atchison 1980; Mukherjee and Sarkar 1981; Walter and Blobel 1983). To address the effect of EDTA on reconstitution of telomerase activity, we first treated partially purified active telomerase fractions with Ca^{2+} and MNase to remove endogenous telomerase RNA. After incubation, MNase was inactivated by chelating Ca^{2+} with EGTA. Following nuclease digestion no telomerase RNA could be detected by Northern analysis (data not shown) and telomerase activity was abolished (Fig. 1; Greider and Blackburn 1987). Activity was restored by incubating MNase-treated telomerase with 5 mM EDTA and 100 ng of in vitro transcribed telomerase RNA, followed by the addition of Mg^{2+} . Reconstitution of telomerase activity specifically required the addition of *Tetrahymena* telomerase RNA. Activity was not restored without RNA nor with several nonspecific RNAs. The addition of excess amounts of nonspecific RNA did not lower the requirement for telomerase RNA (Fig. 1).

To investigate the role of RNA secondary structure on reconstitution of telomerase activity, telomerase RNA was heated to 65°C and cooled slowly to room temperature before adding it to reconstitution reactions. This treatment did not stimulate or inhibit the level of reconstituted activity (data not shown). Reconstitution of telomerase activity was optimal at 37°C for short incubation times (5–10 min). Longer incubation times (15–20 min) or incubation at lower (30°C) or higher (42°C) temperatures decreased the levels of reconstituted telomerase activity (data not shown). Pretreatment of the extract with proteinase K abolished reconstitution of telomerase activity indicating that a protein component(s) is required for reconstitution of activity (Fig. 1).

Reconstitution of primer specificity and cleavage

To further characterize reconstituted enzyme activity we tested the primer specificity of this activity. No elongation of a nontelomeric pBR primer (Fig. 1; Harrington and Greider 1991) was detectable with the reconstituted extract, and no products were generated in the absence of a specific primer oligonucleotide (Fig. 1). This specificity for a telomeric primer is identical to that seen with native telomerase (Greider and Blackburn 1985). Moreover, incorporation of [α - ^{32}P]dGTP onto the telomeric primer d(GGGGTT)₃ was sensitive to pretreatment with RNase A (data not shown) as expected for telomerase (Greider and Blackburn 1987).

Elongation of telomeric primers by *Tetrahymena* te-

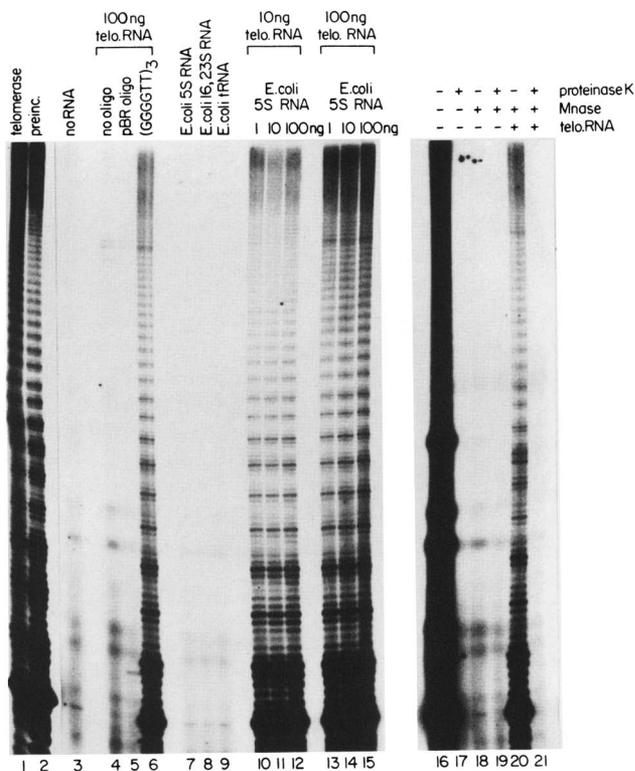


Figure 1. Reconstitution of telomerase activity after MNase treatment. Telomerase assays were performed with telomerase pretreated as indicated. (Lane 1) DEAE-purified telomerase; (lane 2) DEAE-purified telomerase, preincubated under similar buffer conditions as reconstitution reactions. (Lanes 3–15) Telomerase reconstituted with various RNAs—without RNA (lane 3), with 10 ng of telomerase RNA (lanes 10–12), or with 100 ng of telomerase RNA (lanes 4–6, 13–15, 20, 21). *E. coli* 5S rRNA (Boehringer Mannheim) was added as a nonspecific competitor: 1 ng (lanes 10, 13), 10 ng (lanes 11, 14), or 100 ng (lanes 12, 15). Control reconstitution assays with nonspecific RNAs: 100 ng of *E. coli* 5S rRNA (lane 7), *E. coli* 16 and 23S rRNA (Sigma; lane 8), or *E. coli* tRNA (lane 9). Oligonucleotides used in the elongation reactions were d(GGGGTT)₃ (lanes 1–3, 6–21), no oligonucleotide (lane 4), pBR oligonucleotide (sequence, 5'-AGCCACTATCGACTACGCGATCAT-3'; Harrington and Greider 1991; lane 5). (Lanes 16–21) Telomerase pretreated with proteinase K as described. Lanes 1 and 2 are from a lighter exposure than the other lanes (20 hr vs. 14 days). In equivalent exposures these lanes were too dark to distinguish the banding pattern.

telomerase generates a distinct 6-nucleotide repeat pattern. A strongly labeled product within this repeat corresponds to a pause after the addition of the first G residue in the sequence d(GGGGTT)_n (Greider 1991a). The sequence at the 3' end of telomeric primers specifies the first nucleotide added in elongation reactions with telomerase and the number of nucleotides added before pausing occurs (Greider and Blackburn 1987). To confirm that the reconstituted activity was attributable to telomerase, and to test the telomeric primer specificity of the reconstituted activity, five different 18-base oligonucle-

otide permutations of the sequence d(GGGGTT)₃ were used in the elongation assay (Fig. 2A). A light exposure of the data is shown so that the initial repeats in lanes 4–18 can be clearly distinguished. A darker exposure of the d(GGGGTT)₃ elongation products is shown in lanes 19–21 to illustrate that long products are generated in these reactions. Figure 2B illustrates the products expected from the predicted alignment of the primers with the telomerase RNA template. Elongation of the telomeric primer d(GGGGTT)₃ by reconstituted telomerase paused after the addition of the first dG (+1) in the sequence GGGGTT, and every 6 nucleotides after the first dG (+7), as expected for telomerase. Elongation of the telomeric primer d(GGGTTG)₃ generated a strongly labeled product corresponding to the addition of 6 nucleotides, GGGTTG (+6) to the 3' end of the primer, representing the first G residue in the sequence GGGGTT. There was also a labeled product at the position corresponding to the size of the input telomeric primer (P), because of the cleavage activity of telomerase (Collins and Greider 1993; see below). Elongation of the permuted primers d(GGTTGG)₃, d(GTTGGG)₃ and d(TTGCGG)₃ by reconstituted telomerase also generated the correct sequence specific pauses expected for native telomerase and in each case the position of the strongly labeled product was consistent with pausing at the first G residue in the sequence GGGGTT.

Dideoxynucleotides (ddNTPs) were used to determine the nucleotide sequence added onto the telomeric primers (Fig. 2). Substitution of ddTTP for dTTP in elongation assays generated specific chain termination products. Elongation of the permuted telomeric primers d(GGGGTT)₃, d(GGGTTG)₃, d(GGTTGG)₃, and d(GT-TGGG)₃ was terminated after the addition of 5, 4, 3, and 2 nucleotides, respectively. The lengths of the products generated upon incorporation of ddTTP suggested that reconstituted telomerase has similar primer specificities to native telomerase. As a control for ddTTP addition in these experiments, ddCTP was added to telomerase assays. The addition of low concentrations of ddCTP does not alter native telomerase activity as there are no G residues in the wild-type template region (Greider and Blackburn 1985), and similarly had no effect on reconstituted enzyme activity.

Tetrahymena telomerase possesses a specific nucleolytic activity that cleaves primers containing the telomeric repeat d(GGGTTG) at the 3' end (Collins and Greider 1993). The G residue at the 3' end of these primers can align with the C residue at the extreme 5' end of the RNA template. During cleavage, the 3'-terminal G residue is removed and a single [α -³²P]dGTP is added. The primer specificity of the telomerase cleavage reaction is reconstituted with synthetic telomerase RNA (Fig. 2A).

Reconstitution of telomerase activity with mutant RNA template sequences

To determine whether mutant RNAs could be used in the reconstitution assay and to dissect the role of the

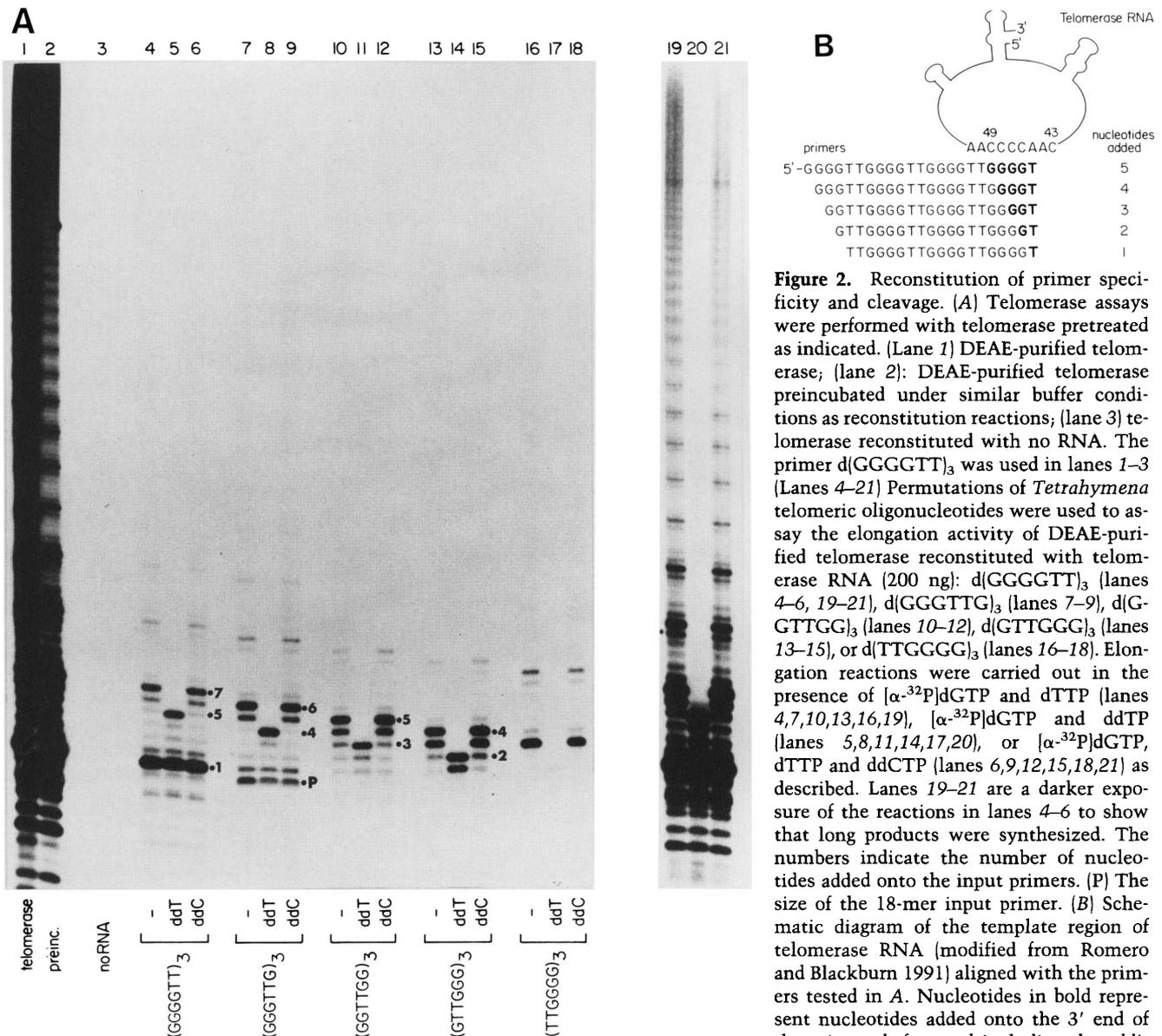


Figure 2. Reconstitution of primer specificity and cleavage. (A) Telomerase assays were performed with telomerase pretreated as indicated. (Lane 1) DEAE-purified telomerase; (lane 2): DEAE-purified telomerase preincubated under similar buffer conditions as reconstitution reactions; (lane 3) telomerase reconstituted with no RNA. The primer d(GGGGTT)₃ was used in lanes 1–3 (Lanes 4–21) Permutations of *Tetrahymena* telomeric oligonucleotides were used to assay the elongation activity of DEAE-purified telomerase reconstituted with telomerase RNA (200 ng): d(GGGGTT)₃ (lanes 4–6, 19–21), d(GGGTTG)₃ (lanes 7–9), d(GGTTGG)₃ (lanes 10–12), d(GTTGGG)₃ (lanes 13–15), or d(TTGGGG)₃ (lanes 16–18). Elongation reactions were carried out in the presence of [α -³²P]dGTP and dTTP (lanes 4,7,10,13,16,19), [α -³²P]dGTP and ddTTP (lanes 5,8,11,14,17,20), or [α -³²P]dGTP, dTTP and ddCTP (lanes 6,9,12,15,18,21) as described. Lanes 19–21 are a darker exposure of the reactions in lanes 4–6 to show that long products were synthesized. The numbers indicate the number of nucleotides added onto the input primers. (P) The size of the 18-mer input primer. (B) Schematic diagram of the template region of telomerase RNA (modified from Romero and Blackburn 1991) aligned with the primers tested in A. Nucleotides in bold represent nucleotides added onto the 3' end of the primers before and including the addition of ddTTP.

tion of ddTTP. For simplicity the primer d(GGGGTT)₃ was aligned as shown. More correctly, the primer should be aligned at the 5' end of the template region, a G residue added, and then aligned as shown subsequent to a translocation of the primer from the 5' end of the template region to the 3' end.

template region in the recognition and elongation of telomeric substrates, five mutant DNA templates were constructed. The template region of the cloned *Tetrahymena* telomerase RNA gene was altered by site-directed mutagenesis of a C → T residue at positions 43, 49, and both 43 and 49. The resulting RNA template sequences were 5'-UAACCCCAA-3' (43U), 5'-CAACCCUAA-3' (49U), and 5'-UAACCCUAA-3' (4349U). These three mutants were initially used because they could potentially specify the synthesis of vertebrate d(TTAGGG)_n telomeric repeats and *Tetrahymena* telomerase will bind and elongate primers of this sequence (Greider and Blackburn 1987). Two additional template mutants were

constructed by changing the A residues at positions 44 and 45 or positions 50 and 51 to G residues. The RNA template sequences were 5'-CGGCCCAA-3' (4445G) and 5'-CAACCCCGG-3' (5051G).

Three mutant RNAs (43U, 49U, and 4349U) reconstituted telomerase activity when assayed in the presence of [α -³²P]dGTP, dATP, dTTP, and d(GGGGTT)₃ (Fig. 3A). However, in the absence of dATP, elongation was most significantly impaired by telomerase enzyme reconstituted with 4349U mutant RNA suggesting that one or both U residues are required for template-directed synthesis of telomeric repeats. Elongation was more processive with telomerase reconstituted with 4349U RNA

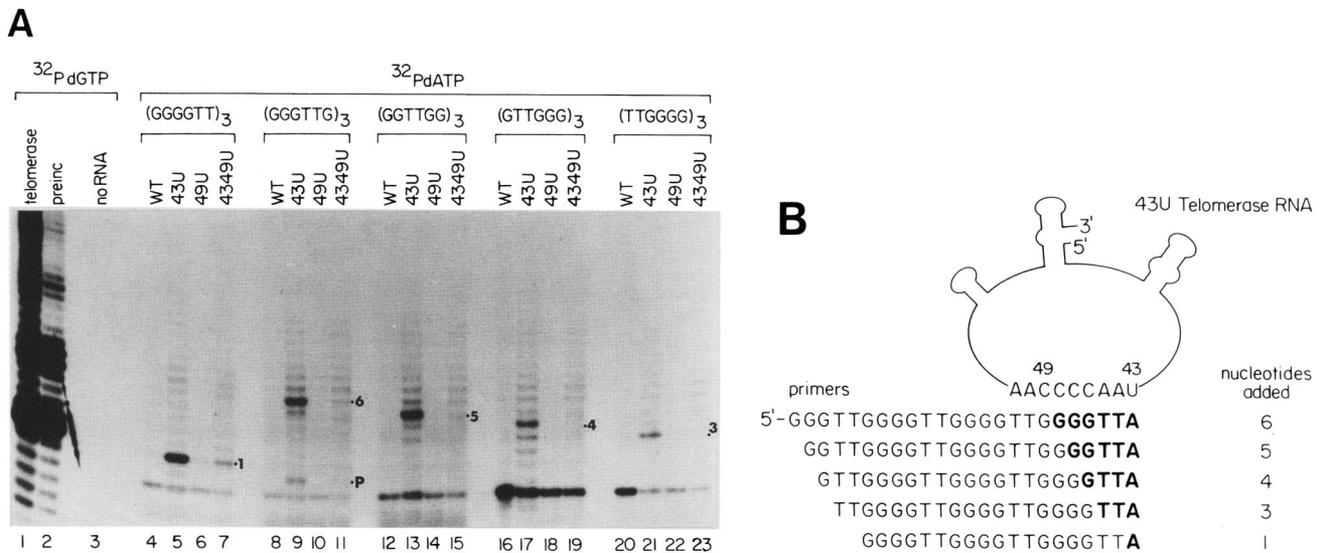


Figure 4. Incorporation of [α - 32 P]dATP by telomerase reconstituted with mutant telomerase RNAs. (A) Telomerase assays were performed with telomerase pretreated as indicated. (Lane 1) Phenyl-Sephadex-purified telomerase; (lane 2) phenyl-Sephadex-purified telomerase preincubated under similar buffer conditions as reconstitution reactions; (lane 3) telomerase reconstituted with no RNA. The primer d(GGGGTT) $_3$ and [α - 32 P]dGTP were used in telomerase reactions in lanes 1–3. Telomerase reconstituted with 200 ng of wild-type (WT) telomerase RNA (lanes 4,8,12,16,20), 43U RNA (lanes 5,9,13,17,21), 49U RNA (lanes 6,10,14,18,22) or 4349U RNA (lanes 7,11,15,19,23), using primers d(GGGGTT) $_3$ (lanes 4–7), d(GGGTTG) $_3$ (lanes 8–11), d(GTTGG) $_3$ (lanes 12–15), d(GTTGGG) $_3$ (lanes 16–19), or d(TTGGGG) $_3$ (lanes 20–23) in elongation reactions. The numbers refer to the number of nucleotides added onto the input primers (P). The labeled product of 17 bases was a nonspecific contaminant. (B) Schematic diagram of predicted alignments between 43U telomerase RNA and telomeric primers. Nucleotides in bold represent nucleotides added before and including the incorporation of [α - 32 P]dATP.

tuted by 49U RNA (data not shown). Enzyme reconstituted with the 43U mutant RNA specified the incorporation of dATP at the correct position within the telomeric repeat (Fig. 4A and B). The d(GGGGTT) $_3$ primer apparently aligned with the template such that the first nucleotide added ([α - 32 P]dATP; +1) was template-directed by the U at position 43 in the RNA. The position of strong pausing or incorporation of [α - 32 P]dATP was 6 nucleotides (+6) from the 3' end of the telomeric primer d(GGGTTG) $_3$. Similarly, a predominant [α - 32 P]dATP-labeled product was present at positions 5, 4, and 3 nucleotides from the 3' end of the primers, d(GTTGG) $_3$, d(GTTGGG) $_3$, and d(TTGGGG) $_3$, respectively. A strongly labeled product was also present at a position corresponding to the size of the input d(GGGTTG) $_3$ primer (P), presumably because of primer cleavage at the 3' terminus and addition of [α - 32 P]dATP. With all oligonucleotide permutations, a less intense product was generated at the position of [α - 32 P]dATP incorporation by telomerase reconstituted with 4349U RNA compared with 43U, suggesting that pausing or dissociation occurs less frequently in this mutant. The experiments illustrated in Figures 3 and 4 indicate that dATP is incorporated into elongation products generated by telomerase reconstituted with 43U and 4349U RNA, but not 49U, suggesting that position 43 of the telomerase template region specifies the incorporation of nucleotides into telomeric DNA products.

When d(GTTGGG) $_3$ was used as a primer in telo-

merase assays with [α - 32 P]dGTP, elongation by telomerase reconstituted with 49U or 4349U RNA was not as efficient when compared with activity reconstituted with wild-type or 43U RNA (data not shown). Inefficient elongation of d(GTTGGG) $_3$ by telomerase reconstituted with 49U or 4349U RNA suggested that mismatches, alignment of primer sequences at more than one position within the template, and/or template slippage might be occurring. Two sites have been proposed for the recognition of telomeric primers (Collins and Greider 1993). One site, presumed to reside in the template region of the RNA, binds the most 3' telomeric repeat of the primer or product DNA. The other site may be a protein component which binds residues 5' of, and adjacent to, the sequences bound to the RNA. This model predicts that changing the template sequence of the RNA would alter the binding specificity to the RNA but not to the protein. To optimize primer binding and alignment of telomerase reconstituted with 49U or 4349U RNA, the chimeric oligonucleotide, d(GTTGGG) $_2$ GTTAGG, was used in telomerase assays. This oligonucleotide has wild-type *Tetrahymena* telomeric repeat sequences at the 5' end for wild-type binding specificity to the protein and mutant *Tetrahymena* telomeric repeat sequences at the 3' end for binding to mutant template RNA sequences. Telomerase reconstituted with 49U or 4349U RNA elongated this oligonucleotide efficiently. Telomerase reconstituted with wild-type or 43U RNA did not elongate this oligonucleotide efficiently (data not

shown), presumably because of mismatches that would occur between the template region of these RNAs and sequences at the 3' end of this primer. Thus, in the experiments described below d(GTTGGG)₃ was used to assay telomerase reconstituted with wild-type or 43U RNA, and d(GTTGGG)₂GTTAGG was used to assay telomerase reconstituted with 49U or 4349U RNA.

To determine the sequence of the nucleotides added by telomerase reconstituted with 43U, 49U, and 4349U RNAs, telomerase reactions were performed using d(GTTGGG)₃ or d(GTTGGG)₂GTTAGG, [α -³²P]dGTP and either ddATP and dTTP or ddTTP and dATP. As a control, reactions were performed with [α -³²P]dGTP, ddCTP, dATP, and dTTP (Fig. 5A). The products of these reactions were compared to elongation of d(GTTGGG)₃ or d(GTTGGG)₂GTTAGG in the presence of [α -³²P]dGTP, dATP, and dTTP. Elongation by telomerase reconstituted with wild-type RNA was not inhibited by ddATP, as expected, as there are no U residues in the wild-type template region. Incorporation of ddTTP occurred 2 nucleotides from the 3' end of the primer at the correct position (see Fig. 2B). Telomerase reconstituted with 43U RNA incorporated both ddATP and ddTTP, 4 and 2 nucleotides from the 3' end of the primer, at the expected positions if the presumed template region, including position 43, was specifying the nucleotides added. In the control reactions the addition of ddCTP did not alter telomerase activity. Using the chimeric oligonucleotide, elongation by 49U mutant telomerase was not inhibited by ddATP, suggesting that position 49 of the template region may not serve as a template (Fig. 5A). Using telomerase reconstituted with 4349U RNA, elongation in the presence of ddATP was terminated 4 nucleotides (+4) from the 3' end of the primer, as expected if position 43 is serving as a template. Incorporation of ddTTP occurred 2 nucleotides from the 3' end of the primer, as expected from the predicted alignment of this primer with the 49U and 4349U RNA templates. Elongation was not inhibited in the presence of ddCTP. These data suggest that, under the conditions described, position 43 but not 49 in the template region of telomerase RNA dictates the addition of nucleotides onto telomeric substrates.

Further evidence for functional specialization within the template region was obtained using RNA mutant 4445G (5'-CGGCCCAA-3') or 5051G (5'-CAACCCCGG-3') (Fig. 5B,C). To determine whether the mutant G residues specified the incorporation of dCTP, telomerase reactions were performed in the presence or absence of either dCTP or ddCTP. In addition, ddTTP was used to determine the template positions at which dTTP was specified (Fig. 5B). Telomerase reconstituted with the 4445G mutant generated a 5 nucleotide addition product using the primer d(GTTGGG)₃ and either [α -³²P]dGTP and dCTP or [α -³²P]dGTP, dCTP, and dTTP (Fig. 5B lanes 6,7). However, when dCTP was omitted full-length elongation was not observed (Fig. 5B, lane 5). This suggested that dTTP was not necessary for elongation to the end of the template; however, dCTP was required. The strong pause or termination after elongation

to the end of the template (+5) may be attributable to inefficient translocation because of the mismatches generated between the newly formed DNA product and the RNA template region. The 4445G mutant telomerase incorporated ddCTP, 3 nucleotides (+3) from the 3' end of the primer (Fig. 5B, lane 9). Some readthrough to position +5 was also observed, probably because of some contamination of the reaction with dCTP. However, no incorporation of ddTTP was observed (Fig. 5B, lane 8). If the A residues at position 50 or 51 were template nucleotides, ddTTP should be incorporated by telomerase reconstituted with 4445G RNA. These data suggest that the G residues at positions 44 and 45 dictate the addition of nucleotides but that the A residues at positions 50 and 51 do not.

The pattern of d(GTTGGG)₃ elongation with the 5051G mutant further supported the interpretation of specialization within the template region. The overall level of elongation with this mutant is low probably attributable to the T-G mismatch in the initial alignment of primer and template (Fig. 5C). Elongation of d(GTTGGG)₃ by the 5051G mutant telomerase with [α -³²P]dGTP in the absence or presence of dCTP generated products with up to 5 residues added onto the 3' end (Fig. 5B, lanes 1,2). When ddTTP was added, elongation terminated after addition of only 3 residues (Fig. 5B, lane 3), as expected if the residue at position 45 is dictating the addition of dTTP. However, no incorporation of ddCTP was observed (Fig. 5B, lane 4), again suggesting that positions 50 and 51 of the telomerase RNA do not serve as template nucleotides.

Discussion

Reconstitution of telomerase activity

Telomerase is an unusual RNP polymerase that plays a vital role in the maintenance of chromosome ends. To understand in detail the mechanism of this RNP we have developed a functional reconstitution assay. Partially purified *Tetrahymena* telomerase was treated with MNase to remove the endogenous telomerase RNA component. Activity was reconstituted by the addition of EDTA and in vitro-transcribed telomerase RNA (Fig. 1). Studies on ribosomes suggest that EDTA does not cause the disassembly of ribosomes into RNA and protein, because after treatment the RNA and protein cosediment on sucrose gradients. Rather EDTA alters the specificity of the protein-RNA interaction such that proteins are free to move from one RNA strand to another (Newton et al. 1975). Thus, the addition of EDTA during reconstitution of telomerase activity may stimulate the interaction between inactivated MNase-treated telomerase and exogenously added synthetic telomerase RNA. Upon the addition of Mg²⁺, protein and RNA components may associate into an active complex. Reconstituted telomerase activity had similar properties to native telomerase activity including processivity, primer specificity, and RNase A sensitivity (Greider and Blackburn 1985). The fidelity of reconstituted enzyme activity suggests

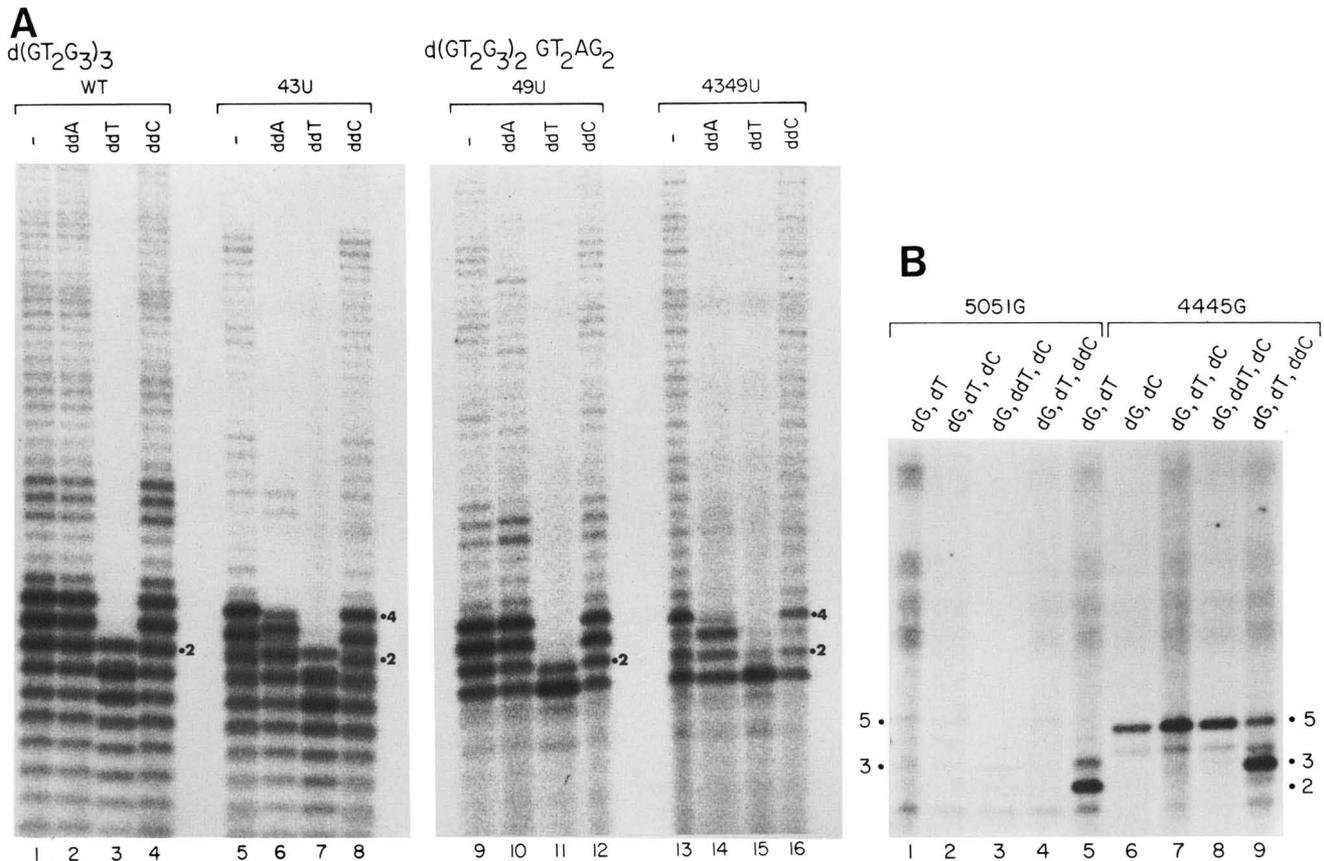


Figure 5. (A) Chain termination upon incorporation of ddATP. Telomerase assays were performed with telomerase pretreated as indicated. Telomerase reconstituted with 200 ng of wild-type telomerase RNA (lanes 1–4), 43U RNA (lanes 5–8), 49U RNA (lanes 9–12), or 4349U RNA (lanes 13–16). Elongation reactions were done using primers $d(GTTGGG)_3$ (lanes 1–8) or $d(GTTGGG)_2GTTAGG$ (lanes 9–16) in the presence of $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$, dTTP, and dATP (lanes 1,5,9,13), $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$, dTTP, and ddATP (lanes 2,6,10,14), $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$, dATP, and ddTTP (lanes 3,7,11,15), or $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$, dTTP, dATP, and ddCTP (lanes 4,8,12,16) as described. Reconstitution reactions shown in lanes 1–8 and 9–16 were performed with phenyl–Sepharose-purified and DEAE-purified telomerase, respectively. The exposure shown in lanes 1–16 is of a PhosphorAutoradiograph (Fujix BAS 2000 BioImaging Analyzer) photograph from a HQ printer (Fuji). The numbers refer to the number of nucleotides added onto the input primers (P). (B) Elongation products of telomerase reconstituted with 4445G and 5051G RNAs. (Lanes 1–9) Telomerase reconstituted with 200 ng of 5051G telomerase RNA (lanes 1–4) and 4445G RNA (lanes 5–9). Elongation reactions were done using primer $d(GGTTGG)_3$ in the presence of $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$ and dTTP (lanes 1,5), $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$ and dCTP (lane 6), $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$, dTTP, and dCTP (lanes 2,7), $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$, dCTP, and ddTTP (lanes 3,8), or $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$, dTTP, and ddCTP (lanes 4,9) as described. Reconstitution reactions were performed with phenyl–Sepharose-purified telomerase concentrated on a DEAE–agarose column. (C) The potential alignment of $d(GGTTGG)_3$ primer with the mutant RNA templates 4445G and 5051G is shown. The effects of adding dTTP, ddTTP, dCTP, and ddCTP on the elongation of this primer are diagrammed. Data are from B.

that the reconstitution assay will be a useful biochemical tool for dissecting native telomerase function. Reconstitution of RNPs generally requires the addition of stoichiometric amounts of RNA and protein nor-

mally found in native complexes (Traub and Nomura 1969; Nierhaus and Dohme 1974; Mukherjee and Sarkar 1981; Walter and Blobel 1983). The kinetics and thermodynamics of the protein–RNA interactions in telomerase

are not yet defined. A typical *in vitro* telomerase reaction contains ~1 ng of telomerase RNA (Avilion et al. 1992). Reconstitution of telomerase activity, however, required the addition of ~100-fold more synthetic telomerase RNA over endogenous telomerase RNA. There are several possible explanations for this requirement: lack of potential RNA base modifications of *in vitro*-transcribed telomerase RNA, inappropriate conformation of the *in vitro*-transcribed telomerase RNA, and/or nonspecific binding of RNA to MNase (Wang and Gegenheimer 1990).

In other studies, RNPs have been reconstituted with both endogenous and *in vitro*-transcribed RNA. Reconstitution of 16S ribosomal subunits from *Escherichia coli* with RNA prepared *in vitro* and lacking modifications normally contained in 16S RNA reduces activity by half (Cunningham et al. 1991). *Tetrahymena* telomerase RNA may be modified (Greider and Blackburn 1989). A lack of modifications of *in vitro*-transcribed telomerase RNA could account for the required concentrations of synthetic RNA for reconstitution of telomerase activity and for the inability, under the conditions described, to restore activity to native levels.

The requirement for 100-fold excess telomerase RNA in reconstitution reactions may also be attributable to the RNA conformation. *In vitro* assembly experiments have shown that correct folding of rRNA depends on sequential, cooperative interactions with ribosomal proteins (Held et al. 1973; Röhl and Nierhaus 1982). Many features of the higher-order structure of 16S rRNA in 30S ribosomal subunits do not exist in 16S rRNA in its naked form (Moazed et al. 1986). Most of the *in vitro*-transcribed telomerase RNA added to the reconstitution reactions may assume incorrect conformations that are incapable of associating with telomerase protein(s).

Protein requirement for reconstitution of telomerase activity

The proteinase K sensitivity of reconstituted telomerase suggests that proteins are required for reconstitution of telomerase activity (Fig. 2). Whether the telomerase RNA component serves a catalytic or structural role is not yet known. Recent experiments provide evidence that 23S rRNA may catalyze the peptidyl transferase reaction of ribosomes (Noller et al. 1992). Ribosomal proteins are important to achieve proper folding and function of rRNA (Held et al. 1973; Röhl and Nierhaus 1982; Stern et al. 1989). Methods used previously to prepare protein-free rRNA to determine whether rRNA was sufficient for peptidyl transferase activity disturbed the higher order structure of the rRNA presumably required for this activity (Noller et al. 1992). RNase P activity can be reconstituted without protein *in vitro* under appropriate ionic conditions; however, protein components are required *in vivo*, to provide stability, functional binding, and assembly or folding of rRNA (Guerrier-Takada et al. 1983). Thus, although studies to date indicate that protein is essential for native and reconstituted telomerase activity, a direct catalytic role for telomerase RNA in

telomere DNA synthesis remains a distinct possibility (Greider and Blackburn 1985, 1987).

Telomerase RNA mutants define distinct functional domains of the RNA template region

All five telomerase RNA template mutants were functional in the reconstitution of telomerase activity (Figs. 3A and 5B). Elongation by telomerase reconstituted with the 4349U mutant RNA was more processive than with the single 43U or 49U mutants; presumably, this efficiency results from having a single mismatch initially between the primer sequence and the RNA template at position 49, and after translocation, no further mismatches (Fig. 3B). In contrast, mismatches would occur repeatedly after each translocation between the 43U or 49U mutant templates and the primer or product sequences. Presumably for this reason, the elongation activity of telomerase reconstituted with 43U or 49U RNA was not as processive as with 4349U RNA.

The reconstitution experiments suggest that the 9 nucleotides of the template region of the telomerase RNA may not all be used as template residues in the elongation of telomeric sequence primers (Figs. 4 and 5). Incorporation of [α - 32 P]dATP by telomerase reconstituted with 43U or 4349U RNA indicated that position 43 of these RNAs serves as a template (Fig. 4A). The position of incorporation of [α - 32 P]dATP from the primer 3' end by telomerase reconstituted with 43U RNA was dependent on the sequence at the 3' end of the primers, as predicted by the primer alignment in Figure 4B. Moreover, the nucleotide added to the cleaved d(GGGTTG)₃ primer was dependent on the changed template nucleotide at position 43. Presumably after cleavage of the 3'-terminal G residue, template-directed addition of [α - 32 P]dATP generated a labeled product corresponding to the size of the input primer. Chain termination by ddATP or ddTTP, at the predicted positions, further confirmed that the mutant region of 43U was serving as a template (Fig. 5A). However, telomerase reconstituted with 49U RNA did not incorporate [α - 32 P]dATP at the concentrations tested, suggesting that position 49 in the template region of telomerase RNA does not serve as a template but may serve to align primer and product sequences (Fig. 4A). Therefore, telomerase reconstituted with 49U would not be expected to incorporate ddATP. Although ddATP was poorly incorporated by telomerase reconstituted with 49U RNA, elongation of the telomeric primer d(GTTGGG)₃ was partially inhibited, indicating that under certain conditions position 49 may serve as a template (data not shown). Further experiments with RNA mutants 4445G and 5051G indicate that positions 44 and 45 also serve as template residues and that positions 50 and 51 probably do not (Fig. 5B). Elongation by telomerase reconstituted with 4445G RNA was dependent on dCTP and not dTTP and was terminated by the addition of ddCTP and not ddTTP, suggesting that positions 44 and 45 but not 50 and 51 direct the addition of nucleotides. Similarly, elongation by telomerase reconstituted with 5051G RNA was inde-

pendent of dCTP and was terminated by the incorporation of ddTTP but not ddCTP, reinforcing the role of positions 44 and 45, but not 50 and 51, as template nucleotides.

Recent experiments suggest that both RNA and protein sites are involved in telomeric primer binding and recognition by telomerase (Collins and Greider 1993). Both sites may be necessary for binding and alignment of primers. Using a chimeric primer, d(GTTGGG)₂GTTAGG, that may ensure optimal binding and alignment to both of these sites in the mutants, elongation by telomerase reconstituted with the 49U and 4349U mutants generated a distinct product pattern and was more efficient than elongation of *Tetrahymena* telomeric sequence primers. Telomerase activity reconstituted with 49U RNA did not incorporate ddATP during elongation of the chimeric primer, suggesting that position 49 in the telomerase RNA does not serve as a template under these conditions (Fig. 5A). Elongation by telomerase reconstituted with 4349U was terminated because of incorporation of ddATP template-directed by position 43. The elongation products of telomerase reconstituted with either mutant 4445G or 5051G and assayed in the presence of various combinations of dTTP, dCTP, ddCTP, and ddTTP clearly indicate that positions 50 and 51 of the telomerase RNA do not serve as template nucleotides (Fig. 5B). We propose a model in Figure 6 that divides the template region into two functionally distinct regions. In this model, the most 5' six residues of the telomerase RNA template region, including the residues at positions 43, 44, and 45 direct the addition of telomeric repeat sequences, whereas the most 3' three residues (49, 50, and 51) of the template region, may serve only to align substrate or product sequences. We refer to the most 5' six residues as the "template" and the most 3' residues as the "alignment region." Experi-

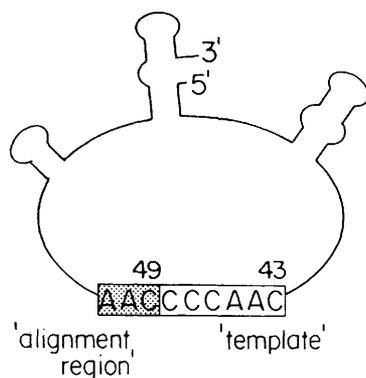


Figure 6. Model for the functional domains of the telomerase RNA template region. The telomerase RNA is shown schematically with the template region 5'-CAACCCCAA-3'. The most 5' six residues (white box) termed the template direct synthesis of telomeric repeats. The most 3' three residues (stippled box) termed the alignment region direct the alignment of substrate and product DNA sequences. However, under suboptimal conditions, as discussed in the text, position 49 may be forced to serve as a template position.

ments with an RNA template region mutant (5'-CCACCCCAA-3') in vivo showed that position 44 serves as a template to specify the sequence added by telomerase (Yu et al. 1990), consistent with the hypothesis that residues 43–48 direct the addition of sequences. In addition, an RNA mutant with the template region 5'-CAACCCCAA-3' specified the synthesis of telomeric G₅T₂ repeats in vivo, indicating that the most 3' C residue in the template region, now at position 49 because of the additional C residue, directs the synthesis of telomeric repeats (Yu and Blackburn 1991). Both in vivo and in vitro reconstitution experiments using other RNA template mutants will be required to further elucidate the role of the residues in this region.

The ability to reconstitute telomerase activity using inactive protein and mutant telomerase RNA will allow the functional dissection of telomerase. The predicted conserved regions of the secondary structure of *Tetrahymena* telomerase RNAs (Romero and Blackburn 1991) and their importance for function can now be tested directly using reconstitution assays. With the identification of telomerase protein components a thorough understanding of telomerase protein–RNA interactions will be possible.

Materials and methods

Purification of oligonucleotides

Oligonucleotides were synthesized by Operon Technologies (Alameda, CA). Oligonucleotides were resuspended in H₂O treated with diethyl pyrocarbonate (DEPC-treated H₂O) and gel purified. An equal volume of oligonucleotide and deionized formamide were mixed, boiled, cooled on ice, and electrophoresed in 12% polyacrylamide–7 M urea gels. Full-length primer was identified using UV shadowing (Sambrook et al. 1989). The DNA was excised, crushed, and eluted from the gel by incubation overnight, in DEPC-treated H₂O, at 37°C. The material eluted from the gel was desalted using Sep-Pak C-18 (Waters) columns, as per the manufacturer's instructions, dried, and resuspended in DEPC-treated H₂O. Oligonucleotide concentrations were determined spectrophotometrically assuming 1 OD₂₆₀ unit equals 33 μg/ml.

Preparation of *Tetrahymena* telomerase

Tetrahymena telomerase was purified using protocols modified from Greider and Blackburn (1987), Collins and Greider (1993), and L. Harrington and C. Greider, (unpubl.). *Tetrahymena* strains SB210 or CU428 [kindly provided by Eric Henderson (Iowa State University, Ames) and Martin Gorovsky (University of Rochester, NY)] were grown, shaking, at 30°C, to a density of 2.5 × 10⁵ cells/ml in 12 liters of media (2% proteose peptone, 0.2% yeast extract, 10 μM FeCl₃) supplemented with 250 μg/l of both ampicillin and streptomycin. After harvesting, the cells were resuspended in Dryl's starvation media (1.7 mM sodium citrate, 2.4 mM sodium phosphate, 2 mM CaCl₂) and incubated at 30°C, for 18 hr. Cells were harvested and resuspended in 2× the volume of the cell pellet, of either TMG (10 mM Tris-acetate or -HCl at pH 8.0, 1 mM MgCl₂, 10% glycerol) or PMG (10 mM PIPES at pH 7.0, 1 mM MgCl₂, 10% glycerol) with 5–10 mM β-mercaptoethanol, 0.1 mM PMSF, and protease inhibitors (0.25 μg/ml each chymostatin, leupeptin, antipain, and pepstatin;

Sigma). Lysis was enhanced by stirring the cells in this buffer, at 4°C, for 20 min in the presence of 0.2% NP-40 (Sigma). The lysed cells were centrifuged at 30,000 rpm for 1 hr in a SW41 rotor at 4°C, and the supernatant (S100 extract, 120 ml) collected and stored at -70°C after quick freezing in liquid nitrogen.

DEAE-purified telomerase was prepared as follows. Extract S100 (120 ml) was loaded onto a 40-ml heparin-agarose (Bio-Rad) column equilibrated in TMG with β -mercaptoethanol and protease inhibitors as described above. Proteins were eluted in TMG with 0.2 M potassium glutamate (KGlu) and frozen in liquid nitrogen. Fractions containing maximal telomerase activity were loaded on an 8 ml DEAE-agarose (Bio-Rad) column equilibrated in TMG plus 0.2 M KGlu. Proteins were eluted in TMG with a 40-ml gradient from 0–0.6 M KGlu. Fractions with maximal telomerase activity (0.3–0.4 M KGlu) were pooled (6 ml), aliquoted, frozen in liquid nitrogen and stored at -70°C. DEAE-purified telomerase (1.3 mg of protein/ml extract) was diluted 10-fold with TMG before use in reconstitution reactions.

Alternatively, the S100 extract (120 ml) was loaded directly onto a 9-ml DEAE-agarose column equilibrated in PMG with β -me and protease inhibitors, and proteins were eluted in PMG with a 45 ml gradient from 0–0.6 M KGlu. Fractions with maximal telomerase activity (0.3–0.4 M KGlu) were pooled (5.5 ml), adjusted to 0.6 M KGlu, and loaded onto a 5 ml phenyl-Sepharose column (Pharmacia) equilibrated in PMG 0.6 M KGlu. Proteins were eluted with a gradient from PMG 0.6 M KGlu to PM 50% ethylene glycol. Most of the telomerase activity eluted with PM 50% ethylene glycol. Fractions with maximal telomerase activity were pooled (5.5 ml), aliquoted, frozen in liquid nitrogen and stored at -70°C. The phenyl-Sepharose-purified telomerase (1.6 mg of protein/ml of extract) was used 10-fold diluted with PMG in reconstitution reactions. The extract preparations remained active in elongation assays and in reconstitution assays, for at least 17 months when stored at -70°C. Protein concentrations were determined by a Bradford assay with Bio-Rad dye reagent.

Telomerase elongation activity assay

Telomerase assays were done essentially as described previously (Greider and Blackburn 1985). Briefly, 20 μ l of extract was added to 20 μ l of a 2 \times reaction mix. The final concentrations of the components in the assay are 1 \times telomerase buffer (50 mM Tris-acetate or -HCl at pH 8.5, 1 mM spermidine, 5 mM β -mercaptoethanol, and 50 mM KOAc), 800 nM primer oligonucleotide (0.2 μ g), 100 μ M dTTP, and 0.3125 μ M [α -³²P]dGTP (1 μ l of 800 Ci/mmol; NEN). In assays with telomerase reconstituted with mutant RNAs, reaction mixes contained 100 μ M of both dTTP and dATP. Reactions with 2.5 μ M [α -³²P]dATP (4 μ l of 400 Ci/mmol; NEN) contained 100 μ M dTTP and 50 μ M dGTP. In reactions with dideoxynucleotides, ddTTP (100 μ M) replaced dTTP, ddATP (100 μ M) replaced dATP, or ddCTP (20 μ M) was added. Reaction times were 1 hr at 30°C except in the experiment shown in Fig. 2 where the incubation time was 20 min. Reactions were stopped by adding 100 μ l of 21 mM EDTA, 10 mM Tris-HCl at pH 7.5. After phenol extraction, 60 μ l of 2.5 M NH₄OAc, 100 μ g/ml of *E. coli* tRNA (Sigma) was added to the reactions and ethanol precipitated for 60 min at room temperature or overnight at -20°C. Products were centrifuged, and pellets dried and resuspended in formamide with bromophenol blue (BPB) and xylene cyanol (XC) or formamide with XC. Samples were boiled, cooled on ice, and loaded onto 8% polyacrylamide-7M urea gels and electrophoresed at 1500 V for 2 hr using 0.6 \times TBE buffer. Gels were dried and then exposed to film

(XAR5) overnight (light exposure) or 7–14 days (dark exposure), or Fuji PhosphorImager screens, generally overnight.

MNase treatment and reconstitution assay conditions

DEAE- or phenyl-Sepharose-purified *Tetrahymena* telomerase extract was incubated with 1–2 units of MNase/ μ l of extract and 0.1 or 1 mM CaCl₂, for 10 min at 30°C. The MNase was inactivated by adding EGTA (2.5 \times the concentration of added CaCl₂). MNase was prepared by resuspending the lyophilized material (Boehringer Mannheim) in DEPC-treated H₂O, at a concentration of 50 or 100 U/ μ l, and stored in aliquots at -20°C. We found that MNase made in DEPC-treated H₂O, stored in aliquots, thawed, and used only once was the least variable in activity, and this became the preferred method for preparing and storing MNase. Extract treated with MNase was used either immediately or stored, in aliquots, at -70°C (after the addition of EDTA to 5 mM) until use. MNase-treated extract was stable at -70°C for at least 4 months. It was important to use only enough MNase to completely abolish telomerase activity, as initially determined by the titration of extract using freshly diluted and aliquoted MNase. Using an excess of MNase decreased the levels of reconstituted activity. In some experiments, telomerase extract was treated with proteinase K (0.2 μ g/ μ l) for 10 min at 30°C, prior to MNase treatment. In standard reconstitution conditions, MNase-treated telomerase extract (~1.5 mg/ml of protein) was incubated with 5 mM EDTA and 100–200 ng of in vitro-transcribed telomerase RNA per 50 μ l of extract, for 5 min at 37°C. Prior to assaying for elongation activity, 1 μ l of 200 or 500 mM MgCl₂ was added to 20 or 50 μ l of the extract, respectively.

Site-directed mutagenesis

The telomerase RNA gene cloned into pUC119 (pCG1; Greider and Blackburn 1989) was subcloned downstream of the T7 promoter. Briefly, oligonucleotides complementary to the 5' (5' T7) and 3' (3' T7) regions of the RNA gene were used in PCR with pCG1 cleaved with *Hind*III, as the template. The sequences of the 5' T7 and 3' T7 oligonucleotides were 5'-GGGGTCTA-GATAATACGACTCACTATAGGGATACCCCGTTAATTC-ATTCA-3' and 5'-CCCCGGATCCAAAAAATAAGACATCCATTG-3', respectively, and included, for the 5' T7 oligonucleotide, the T7 promoter and a *Xba*I restriction site. PCR amplification was done in a 100 μ l volume in the presence of 1 μ M of each primer and 3 ng of *Hind*III-digested pCG1 plasmid DNA using 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus) in 1 \times PCR buffer (50 mM KCl, 10 mM Tris-HCl at pH 8.3, 3 mM MgCl₂) and 250 μ M dNTPs. Reactions were cycled 16 times for 2 min at 94°C, 3 min at 37°C, and 3 min at 72°C. The PCR-amplified DNA was cleaved with *Xba*I and *Bam*HI and cloned into pUC119 cleaved with *Bam*HI and *Xba*I using conventional cloning techniques (Maniatis et al. 1982). The resulting clone, pT7159, contained the telomerase RNA gene downstream of the T7 promoter, as confirmed by sequencing both strands of the inserted DNA by the dideoxy-mediated chain termination method as per the manufacturer's instructions (U.S. Biochemical).

Using the same methodology, plasmids containing mutant versions of the RNA gene were constructed. Oligonucleotides with mutations complementary to positions 43, 49, both 43 and 49, both 44 and 45, or both 50 and 51 of the template region of the RNA gene were synthesized and used in PCR, with pT7159 digested with *Eco*RI and *Hind*III, as a template. The sequences of the oligonucleotides were 5'-CATTGAGATCT-GTAATAGAACTGTCATTTAACCCCAAAAATC-3' (5'43T),

5'-CATTGAGATCTGTAATAGAAGTGCATTCAACCCT-AAAAATC-3' (5' 49T), 5'-CATTGAGATCTGTAATAGAAGTGCATTCAACCCTAAAAATC-3' (5' 4349T), 5'-CATTGAGATCTGTAATAGAAGTGCATTCAACCCTAAAAATC-3' (4445G), and 5'-CATTGAGATCTGTAATAGAAGTGCATTCAACCCTAAAAATC-3' (5051G). The 3' T7 oligonucleotide used was the same as described above. PCR conditions were as described above except that 1 ng of plasmid pT7159 digested with *EcoRI* and *HindIII* was used. Reactions were cycled 20–30 times for 5 min at 95°C, 1 min at 46°C, and 3 min at 72°C. The amplified DNAs were digested with *BamHI* and *BglIII* (for the 43T, 49T, and 4349T mutants) or *EcoRI* and *BglIII* (for the 4445G and 5051G mutants) and cloned into pT7159 digested with the same enzymes. The resulting clones, pT743T, pT749T, pT74349T, pT74445G, and pT75051G, contained mutant RNA genes, as confirmed by sequencing both strands of the entire gene.

Preparation of RNAs

RNAs used in reconstitution assays were *in vitro* transcribed with T7 RNA polymerase (Boehringer Mannheim; NEB) using pT7159, pT743T, pT749T, pT74349T, pT74445G, and pT75051G digested with *FokI*, as templates. Standard *in vitro* transcription reaction conditions recommended by the T7 RNA polymerase manufacturer were used. The RNAs were either gel purified or the transcription reactions treated with RNase-free DNase (Boehringer Mannheim). The RNA concentrations were determined spectrophotometrically or by fluorometer. The *in vitro* transcribed RNA was 3 nucleotides longer than the endogenous RNA because of the addition of 3 G residues at the 5' terminus.

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