Telomerase as a DNA-Dependent DNA Polymerase†

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ABSTRACT: Telomerase is a specialized reverse transcriptase, which catalyzes the addition of telomeric repeats to the 3′ ends of linear chromosomes using its integral RNA subunit as the template. An active *Tetrahymena thermophila* telomerase complex can be reconstituted *in vitro* from two essential components, tTERT, the catalytic protein subunit, and tTR, the RNA subunit. While the sequence specificity of telomerase has been investigated using template sequence mutants, there is no information regarding its backbone specificity. To address this question, we engineered two mutant forms of the telomerase RNA subunit that contain DNA only in the templating region and used rabbit reticulocyte lysates to reconstitute telomerase activity with the chimeric tTRs. The resultant telomerase mutants were able to extend telomeric DNA primers, albeit with reduced efficiency compared to the wild type. The reduced activity is presumed to be a function of the nascent DNA−template duplex structure. Additionally, the DNA-dependent telomerase mutants were RNase-sensitive, confirming that nontemplate portions of tTR are critical for maintaining activity of the telomerase ribonucleoprotein complex even after it is assembled. The splint ligation approach that we outline will allow the generation of tTR mutants containing a variety of nucleotide analogues, facilitating more elaborate studies of the interactions between the telomerase template and active site.

Telomerase is a specialized reverse transcriptase that is responsible for maintaining telomere length in most eukaryotic cells. Telomerase catalyzes the addition of deoxynucleotide monophosphates to the 3′ ends of linear chromosomes by using a portion of its integral RNA subunit as the template (*1*, *2*). When telomerase extends the 3′ end of the chromosome, it provides a template for the normal DNA replication machinery, allowing the complementary strand of the DNA to be synthesized. This process, which is repeated after DNA replication during every cell cycle in telomerase positive cells, prevents chromosome shortening that would naturally result from the end replication problem and therefore ensures genetic stability.

Telomerase is a ribonucleoprotein complex containing a catalytic protein subunit, telomere reverse transcriptase (TERT),[1] and an integral RNA subunit, telomerase RNA (TR). Even though many organisms require other protein components to allow telomere maintenance *in vivo* (*2*), the minimal requirements for *in vitro* reconstitution of telomerase activity are TERT and TR. These essential telomerase components have been identified in a wide range of organisms (*3*, *4*). Comparative sequence analysis has revealed that TERT is homologous to reverse transcriptases (RTs) and contains several conserved RT motifs that are important for activity (*5*, *6*). This conservation of sequence is not surprising because the primary catalytic function of telomerase is to synthesize DNA using its RNA subunit as the template, which is by definition a reverse transcription reaction (*7*). The key difference between telomerase and other RTs is that the telomerase RNA also serves as a critical structural component of the enzyme, while only a small portion of the RNA subunit is used as the specific template for DNA synthesis. In contrast, RTs can generally utilize any RNA as a template, and the RNA does not typically serve as a structural component of the RT complex.

While the protein subunits of telomerase contain substantial amino acid conservation between different organisms, the RNA subunits vary greatly in size (∼160−1400 nucleotides) and primary sequence (*8*). Recently, it has become apparent that several secondary structure elements of TRs may be conserved (*9*−*12*), including a pseudoknot structure located 3′ of the template (Figure 1A). Other structural features are less conserved but appear to be important for protein binding (*13*, *14*), defining the template boundary (*15*−*17*), DNA synthesis (*13*, *18*), and recruiting other protein components of the telomerase complex (*9*).

The *Tetrahymena thermophila* telomerase is one of the most thoroughly investigated telomerase complexes because of its early discovery (*19*), the relative abundance of the endogenous enzyme (*19*), and the ability to reconstitute robust telomerase activity *in vitro* using the recombinant genes of the *Tetrahymena* TERT (tTERT) and TR (tTR) (*20*, *21*). The *Tetrahymena* telomerase holoenzyme can easily be

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[1] Abbreviations: RRL, rabbit reticulocyte lysate; RT, reverse transcriptase; TERT, telomere reverse transcriptase; TR, telomerase RNA; tTERT, *Tetrahymena thermophila* TERT; tTR, *Tetrahymena thermophila* TR; tTR52−159, the 3′ fragment of tTR from nucleotides 52 to 159; tTRd43−51, tTR with DNA replacing RNA in positions 43−51; tTRd43−48, tTR with DNA replacing RNA in positions 43−48.

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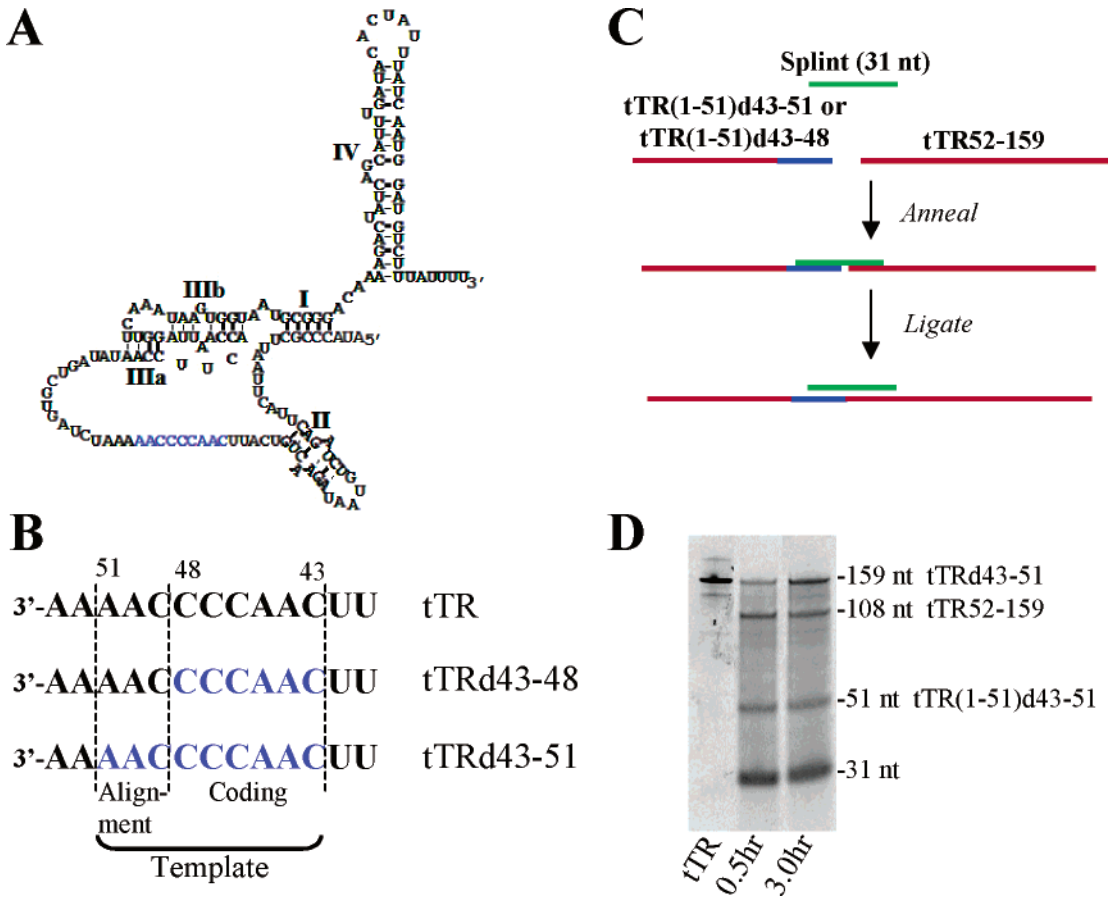

FIGURE 1: *Tetrahymena* telomerase RNA and DNA containing mutants. (A) Proposed secondary structure of tTR [adapted from Chen and Greider (*8*)]. (B) Template regions of tTR and DNA template mutants tTRd43−48 and tTRd43−51 with dNMPs colored blue. (C) Schematic of splint ligation reactions used to produce chimeric tTR mutants. (D) Denaturing PAGE analysis of ligation progression. Aliquots of a small-scale ligation reaction were taken at 30 min and 3 h time points and analyzed by denaturing PAGE and stained with SYBR Green II. Wild-type tTR was included as a reference.

assembled *in vitro* by expressing tTERT in a rabbit reticulocyte lysate (RRL) in the presence of or followed by the addition of *in vitro* transcribed tTR (*20*). Detailed studies relating the primary and secondary structure of tTR with telomerase activity have been performed using the *in vitro* reconstituted *Tetrahymena* telomerase as well as the native complex (*13*, *17*, *22*, *23*). The template region, one of the most studied portions of tTR (*24*−*26*), is defined by nine nucleotides, 3′-^{51}AACCCCAAC^{43}-5′. The first three 3′ nucleotides within the template function in aligning the telomerase primer, while the following six nucleotides function as the coding residues (Figure 1B) (*27*−*29*). Previous studies revealed that telomerase can tolerate many changes within its template while retaining catalytic ability. Specifically, Ware et al. (*30*) showed that telomerase containing a nontelomeric template composed entirely of AU repeats or only poly-U was capable of extending a primer by adding the proper complementary sequence, although the processivity of these template mutants was dramatically reduced. Furthermore, Miller and Collins (*15*) demonstrated that the template could be added entirely *in trans* to the remainder of the RNA subunit and still yield a functional telomerase, although with severely reduced activity. These key studies indicate that, while telomerase is functional with a variety of RNA templates, it is an obligate ribonucleoprotein complex that requires specific elements both within and away from the template to establish full activity. To further the understanding of telomerase as a specialized reverse transcriptase, it is important to examine its template specificity compared to other RTs. For example, can telomerase utilize a DNA template?

In this paper, we outline a strategy to engineer tTR with any nucleotide or nucleotide analogue amenable to synthesis by standard solid-state techniques to be present within the telomerase template (Figure 1C). We used this technology to synthesize two chimeric versions of tTR that contain DNA within the template (Figure 1B). Assembling the resulting mutants with recombinant tTERT allowed us to characterize for the first time the ability of telomerase to function as a DNA-dependent DNA polymerase.

MATERIALS AND METHODS

Oligonucleotides. DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were purified using denaturing PAGE followed by a modified version of the crush and soak method (*31*, *32*). Briefly, after electrophoresis, oligonucleotides were visualized by UV shadowing, gel slices were removed and crushed by passing through the tip of a sterile plastic syringe, and the oligonucleotides were extracted from the gel into TEN buffer (10 mM Tris at pH 7.5, 1 mM EDTA, and 250 mM NaCl). Oligonucleotides were then concentrated by ethanol precipitation and resuspended in TE (10 mM Tris-HCl at pH 8.0 and 1 mM EDTA). RNA oligonucleotides and the RNA−DNA chimeras tTR(1−51)d43−51 and tTR(1−51)d43−48

were purchased from Dharmacon (Lafayette, CO) in the 2′-ACE-protected form. These synthetic RNAs were deprotected following the instructions of the manufacturer and were not further purified before use. All oligonucleotides were analyzed by denaturing PAGE to confirm their purity. Typically, individual sequences were >95% full-length oligonucleotides. Oligonucleotide concentrations were determined by UV absorbance at 260 nm using the molar extinction coefficient provided by the manufacturer.

Plasmids. A construct that contains the tTERT gene with an N-terminal T7 tag inserted into the vector pET-28a (Novagen), pET-28a-tTERT, was a gift from the laboratory of Dr. Thomas R. Cech (*33*). pTET-telo, a plasmid containing the tTR gene, a promoter for T7 RNA polymerase, and a self-cleaving hammerhead ribozyme that processes the 5′ end of the RNA to generate wild-type tTR was a gift from Dr. Art Zaug (*34*).

PCR Amplification of the tTR52−*159 Template.* The template for tTR52−159, the 108-nucleotide 3′ portion of tTR used for splint ligations, was generated by PCR. Briefly, two DNA oligonucleotides, antisense strand 5′-CGGGGATCCTCTTCAAAAATAAGACATCCATTGATAAATAGTGTATCAAATGTCGATAGTCTTTTGTCCCGCATTACCACTTATTTGAACCTAATTGGTGAAGGTTAATCAGC and sense strand 5′-CGCGGAATTCTAATACGACTCACTATAGGGAGGAGA*TTTCTGATGAGGCCGAAAGGCCGAAACTCCACGAAAGTGGAGTAAAA*TCTAGTGCTGATATAACCTTCACCAATTAGG, which are complementary along the underlined portions, were subjected to five cycles of PCR, 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, to yield the full-length, double-stranded product. This product was further amplified by the addition of sense primer 5′-GCGCGGAATTCTAATACGACTCACTATAGG and antisense primer 5′-CGGGGATCCTCTTCAAAAATAAGA using 30 additional PCR cycles. The 204-base-pair PCR product was purified using Wizard PCR Preps DNA Purification System (Promega) following the instructions of the manufacturer. The italic portion of the sense strand codes for a self-cleaving hammerhead ribozyme that generates the appropriate 5′ end required for efficient ligation of the product RNA, tTR52−159.

Transcription of RNAs. Full-length tTR and tTR52−159 were prepared by *in vitro* transcription using the T7 RNA polymerase AmpliScribe kit (Epicentre) and the corresponding linear, double-stranded DNA templates. For full-length tTR, 20 μg of pTET-telo was digested with the restriction enzyme *Ear*I (New England Biolabs), which cuts 3′ of the tTR gene. Digested DNA was deproteinized by phenol/chloroform/isoamyl alcohol extraction, concentrated by ethanol precipitation, and resuspended in TE. The template for the tTR52−159 was prepared by PCR as described above. Both transcription reactions followed the protocol of the manufacturer (Epicentre). After transcription, the reactions were diluted 4-fold into 20 mM $MgCl_2$ to activate hammerhead ribozyme cleavage. After ribozyme cleavage, the RNAs were concentrated by ethanol precipitation and reconstituted in denaturing loading buffer (89 mM Tris, 89 mM boric acid at pH 8.3, 2 mM EDTA, 7 M urea, 10% glycerol, 0.02% bromophenol blue, and 0.02% xylene cyanol FF). RNAs were purified by denaturing PAGE on 1.5-mm thick, denaturing 10% polyacrylamide gels as described above.

Synthesis of tTRd43−*51 and tTRd43*−*48.* Full-length chimeric tTR mutants were synthesized by ligation reactions mediated by DNA splints (*35*). Prior to splint ligation, 750 pmol of tTR52−159 RNA were 5′-phosphorylated with ATP (25 μM) using 150 units of T4 polynucleotide kinase (PNK, New England Biolabs) and PNK buffer at 37 °C for 35 min. The reaction was stopped by heating at 95 °C for 5 min. Excess nucleotides were removed from the reaction using a MicroSpin G-25 column (Amersham Biosciences) per the instructions of the manufacturer. The RNA was deproteinized by phenol/chloroform/isoamyl alcohol extraction, concentrated by ethanol precipitation, and resuspended in a suitable volume of TE. Ligation of 5′-phosphorylated tTR52−159 to tTR(1−51)d43−51 or tTR(1−51)d43−48 was performed by combining 500 pmol of each of the two partnering RNAs along with 600 pmol of a 31-nucleotide DNA splint, 5′-TCAGCACTAGATTTTTGGGGTTGAATGACAG, which is complementary to nucleotides 35−51 of tTR(1−51)d43−48 or tTR(1−51)d43−51 and nucleotides 52−65 of tTR52−159 (see Figure 1C). The 5′ and 3′ tTR components and DNA splint were heated to 95 °C and cooled slowly to 0 °C over 1 h followed by the addition of 0.1 vol of 10× T4 ligase buffer (Promega) and 250 units of T4 DNA ligase (Promega) in a final volume of 500 μL. Ligation reactions were incubated at 37 °C for 3 h after which they were extracted with phenol/chloroform/isoamyl alcohol, concentrated by ethanol precipitation, and resuspended in denaturing loading buffer. The chimeric tTR products were purified by denaturing PAGE as described above (*31*, *32*). Small-scale ligation reactions (10 pmol of total RNA) were performed prior to large-scale reactions to optimize the reaction conditions. Reaction progress was monitored by SYBR Green II stained denaturing polyacrylamide gels of different reaction time points.

5′-^{32}P-Labeling of tTRs. Telomerase RNAs were 5′-end labeled by incubating 2.75 pmol of tTR, tTRd43−51, or tTRd43−48 with 10 units of PNK, 20 pmol of [γ-^{32}P]ATP (6000 Ci/mmol; Perkin−Elmer), and 1× PNK buffer at 37 °C for 35 min. The radiolabeled RNAs were purified using a MicroSpin G-25 column to remove unincorporated nucleotides, extracted with phenol/chloroform/isoamyl alcohol, concentrated by ethanol precipitation, and resuspended in TE. The specific activity of the RNAs was determined by liquid scintillation counting on a Packard 1900 TR liquid scintillation analyzer.

Translation of tTERT and Telomerase Assembly. tTERT was translated and assembled with tTR using a TNT Coupled Reticulocyte Lysate Systems kit (Promega) based on a previously described procedure (*33*). A typical 50 μL reaction contained 1 μg of pET-28a-tTERT, 75 ng of tTR, tTRd43−51, or tTRd43−48, 34 pmol of [^{35}S]methionine (1175 Ci/mmol; Perkin−Elmer), and additional reaction kit components provided by the manufacturer. Reactions were incubated at 30 °C for 90 min and were flash-frozen in an ethanol/dry ice bath and stored at −80 °C. A total of 5 μL of each lysate reaction was analyzed by SDS−PAGE as follows. Samples were denatured by the addition of an equal volume of 2× SDS gel-loading buffer (100 mM Tris-HCl at pH 6.8, 200 mM DTT, 20% glycerol, 4% SDS, and 0.05% bromophenol blue) and were heated at 95 °C for 5 min. The denatured samples were electrophoresed on 6% acrylamide/SDS gels (*36*). Gels were dried before exposing overnight to phos-

phorimager plates, which were imaged on a Molecular Dynamics Storm 860 phosphorimager.

Immunoprecipitation of the Telomerase Complex. To purify the telomerase complex for enzyme assays and to measure the relative amounts of RNA bound to tTERT, we used an immunprecipitation procedure (*33*). Anti-T7 antibody agarose beads (Novagen; 50 μL) were washed 4 times in 750 μL of wash buffer 1 (20 mM Tris-acetate at pH 7.5, 100 mM potassium glutamate, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.1% IGEPAL). Between each step, beads were recovered by centrifugation at 1500*g* for 2 min at 4 °C. The beads were then blocked twice using 500 μL of blocking buffer (wash buffer containing 0.5 mg/mL lysozyme, 0.5 mg/mL BSA, 0.05 mg/mL glycogen, and 0.1 mg/mL yeast RNA) for 15 min at 4 °C with gentle mixing on an orbital shaker. A total of 75 μL of RRL translation reaction containing assembled telomerase was added to 75 μL of blocking buffer and centrifuged at 17000*g* for 10 min at 4 °C to remove any precipitates. The supernatant was then added to 50 μL of blocked T7-agarose beads, and the resultant slurry was mixed on an orbital shaker for 3 h at 4 °C. The beads were washed 4 times with 750 μL of wash buffer 3 (20 mM Tris-acetate at pH 7.5, 300 mM potassium glutamate, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.1% IGEPAL), 2 times with 750 μL of TMG (10 mM Tris-acetate at pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, and 10% glycerol), and resuspended in 50 μL of TMG to afford a 1:1 slurry. Samples were either flash-frozen in an ethanol/dry-ice bath and stored at −80 °C or were prepared for SDS−PAGE analysis as described above.

Telomerase Activity Assay. Telomerase assays contained telomerase from crude RRL reactions (10 μL), telomerase reaction buffer (50 mM Tris-HCl at pH 8.3, 1.25 mM $MgCl_2$, and 5 mM DTT), 2 μM telomeric primer, 100 μM dTTP, 10 μM dGTP, and 0.33 μM [α-^{32}P]dGTP (3000 Ci/mmol) in a final reaction volume of 20 μL. In some experiments, 0.5 μg of DNase-free RNase A was added to the telomerase buffer before the addition of telomerase as a control for RNA-dependent primer extension (*24*). Variations from standard conditions, including primer sequences and variation in nucleotide substrates are described in the appropriate figures and figure captions. Reactions were incubated at 30 °C for 1 h followed by the addition of an 80 μL aliquot of TES stop buffer (50 mM Tris-HCl at pH 8.0, 20 mM EDTA, and 0.2% SDS) containing a 5′-^{32}P-labeled 114-nucleotide oligonucleotide for extraction and loading control. Extension products were purified by phenol/chloroform/isoamyl alcohol extraction and concentrated by ethanol precipitation using 2 M ammonium acetate and 100 μg/mL glycogen as a counterion and carrier, respectively. The resulting pellets were resuspended in denaturing loading buffer and heated to 95 °C for 5 min prior to their separation using denaturing PAGE. Products were resolved on 0.4-mm thick, denaturing 8% polyacrylamide sequencing gels, which were run at 70 W for 1.25 h. Dried gels were exposed overnight to phosphorimager plates. Plates were imaged using a Molecular Dynamics Storm 860 phosphorimager and were analyzed using ImageQuant 5.1.

Thermal Stability of Model Primer·Template Duplex. Duplexes d(CAACCCCAA)/r(UUGGGGUUG), d(CAACCCCAA)/d(TTGGGGTTG), r(CAACCCCAA)/r(UUGGGGUUG), and r(CAACCCCAA)/d(TTGGGGTTG) were annealed at a total strand concentration of 8 μM in 50 mM Tris-HCl at pH 8.3 and 1.25 mM $MgCl_2$ in a final volume of 500 μL. UV absorbance at 260 nm (A_{260}) was monitored on a Perkin−Elmer UV/vis spectrophotometer λ 20 with the temperature controlled by a PTP-6 Peltier System and Fisher Scientific Isotemp 1016S. Temperature was changed at a rate of 2 °C/min from 10 to 75 °C and from 75 to 10 °C in duplicate. Melting temperatures were determined from the first derivative of the A_{260} versus temperature curve.

RESULTS

Synthesis and Characterization of DNA-Containing Mutants of tTR. The sequence specificity of *Tetrahymena* telomerase has been extensively investigated (*24*, *25*, *28*−*30*). However, the ability of telomerase to utilize a DNA template has been more difficult to probe because the role of the RNA subunit in establishing an active telomerase is more complex than merely supplying the template for reverse transcription. To examine the backbone specificity of telomerase, we therefore synthesized two mutants of the *Tetrahymena* telomerase RNA that contain deoxyribonucleotides only within the templating portion. These telomerase RNA template mutants were constructed by splint ligation (Figure 1C) (*35*). In one mutant, tTRd43−48, DNA was substituted into only the coding residues of the template, positions 43−48, while in the other, tTRd43−51, DNA was substituted into the entire template domain, positions 43−51, which includes the alignment region (Figure 1B). To ensure that the ligation proceeded efficiently, samples from a small-scale ligation were removed at varying time points and analyzed by denaturing PAGE. The amount of full-length ligation product increased over 3 h, with further incubation time not affecting the overall yield (Figure 1D). Optimized large-scale ligations typically yielded 50−60% full-length product RNA. Ligation products (tTRd43−48 and tTRd43−51) and *in vitro* transcribed wild-type tTR were sequenced after RT PCR amplification to ensure they were correct, and they were treated with RQ1 DNase to confirm the presence of DNA in the template (see the Supporting Information). We also found that M-MLV RT was able to reverse transcribe across the chimeric tTRs and wild-type tTR with minimal pausing (see the Supporting Information).

Tetrahymena Telomerase Can Assemble with Chimeric tTR. We used a RRL expression system to transcribe and translate tTERT with an N-terminal T7 epitope tag. Telomerase was then conveniently assembled by the addition of tTR or one of the chimeric tTRs to the RRL reaction (*21*). To assess the binding of tTERT to the chimeric tTR mutants, we co-immunoprecipitated 5′-^{32}P-labeled wild-type and chimeric tTRs with ^{35}S-labeled tTERT. We found that both tTRd43−48 and tTRd43−51 associated with tTERT (Figure 2). However, the amount of the chimeric tTRs that co-immunoprecipitated with tTERT was consistently lower than that of wild-type tTR (48 ± 20% for tTRd43−48; 49 ± 23% for tTRd43−51; data from three independent trials). The reduced binding of tTERT to the tTR mutants was unexpected because the presence of a template does not appear to be an important contributor to the stability of the telomerase complex (*15*, *18*). However, we did observe a decreased stability of the chimeric tTRs in the RRL, when compared to tTR (data not shown), suggesting that decreased stability of the mutants could explain the diminished co-

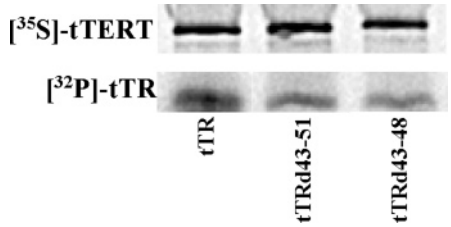

FIGURE 2: Association of chimeric tTR mutants with tTERT. ^{35}S-Labeled tTERT was synthesized in RRL in the presence of 5′-^{32}P-labeled tTR, tTRd43−51, or tTRd43−48. Complexes were immunoprecipitated with anti-T7 agarose beads and resolved on 6% SDS−PAGE gels.

immunoprecipitation efficiency. Controls using expression of an empty vector revealed that co-immunoprecipitation of tTR, and the chimeric tTR mutants required the presence of tTERT (data not shown). Importantly, once assembled into the holoenzyme, the mutant tTRs were stable, because long-term storage (at least 9 months) of assembled mutant telomerase did not result in a noticeable decrease of the enzymatic activity.

DNA-Dependent Telomerase Reaction. Telomerase has two types of catalytic activity, deoxynucleotide addition (*19*) and exo- or endonuclease cleavage (*37*). Telomerase also demonstrates two types of processivity. Type-I processivity, common to all nucleic acid polymerases, is the processive addition of single deoxynucleotides to a primer until the template boundary is reached. Type-II processivity, which is also called repeat addition processivity, describes the synthesis of multiple telomeric repeats, requires primer realignment after extension to the template boundary, and is unique to telomerase (*38*, *39*). We characterized the catalytic properties of telomerase when it utilizes a DNA template by using a telomerase-dependent primer extension assay (*21*). Telomerase reconstituted with either tTRd43−48 or tTRd43−51 (lanes 3 or 4 of Figure 3, respectively) demonstrated reduced overall activity (<10% of the wild-type activity), as measured by total lane density, when compared to telomerase reconstituted with wild-type tTR (lane 1). Furthermore, telomerase reconstituted with either of the chimeric tTR mutants demonstrated no observable type-II processivity. tTRd43−48 telomerase efficiently added only two dGMP residues to the primer, whereas tTRd43−51 telomerase added only one. Neither mutant complex was efficient at the addition of dTMP. Negative controls included RNase treatment (lane 2), in which RNase A was added to the reaction prior to primer addition (*24*), reactions using tTERT assembled with the RNA fragments used to synthesize the chimeric tTRs, tTR52−159 (lane 5), tTR(1−51)-d43−48 (lane 6), or tTR(1−51)d43−51 (lane 7), and reactions containing no tTERT, where an empty plasmid was added to the RRL for assembly with tTRd43−48 (lane 8) or tTRd43−51 (lane 9). These controls demonstrated that the observed activity required full-length tTR or chimeric tTR, as well as the catalytic protein subunit (lanes 5−9).

Substrate K_M Is Not Affected by DNA Template. One explanation for the decreased ability of telomerase to utilize a DNA template could be reduced affinity for its DNA primer (*13*). We therefore examined the effect of the primer concentration on wild-type and chimeric tTR telomerases (Figure 4). Notably, the K_M for the primer was not altered significantly between the wild-type telomerase and the

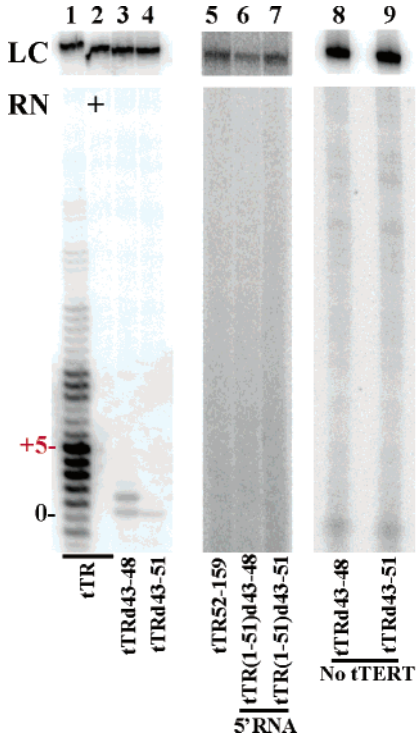

FIGURE 3: *In vitro* reconstitution of telomerase activity with DNA template tTRs. Telomerase was reconstituted *in vitro* in RRL with tTERT and tTR (lanes 1 and 2), tTRd43−48 (lane 3), or tTRd43−51 (lane 4). As a negative control for tTR fragment activity, tTERT was reconstituted in RRL with the 3′ fragment tTR52−159 (lane 5) and the 5′ chimeric fragments tTR(1−51)d43−48 (lane 6) and tTR(1−51)d43−51 (lane 7). As a control for tTERT activity, RRL expressing an empty vector was incubated with tTRd43−48 (lane 8) or tTRd43−51 (lane 9). Crude telomerase complexes in RRL were incubated with primer p5 ($G(T_2G_4)_2TTGG$), dTTP, and [α-^{32}P]-dGTP. The red "5" indicates the 5th nucleotide added to the end of the primer (addition of dGMP across template position 43) as well as the end of the template. The black "0" indicates the length of unextended primer p5. Note that the shift in lanes 2−4 is a gel artifact also reflected in the loading control (LC). A ^{32}P-labeled 114-nucleotide oligonucleotide was used as a recovery and LC. Lanes labeled with RN are assays of samples pretreated with RNase.

chimeric tTR telomerases (0.5 ± 0.06 μM for tTR; 0.9 ± 0.3 μM for tTRd43−48; 0.3 ± 0.1 μM for tTRd43−51; see the Supporting Information). The increase in type-II processivity that was observed in this experiment for tTRd43−48 telomerase at primer concentrations of 2 μM and above was probably related to the overall increase in activity. The concentration dependence of nucleotide substrates dTTP and dGTP was also tested, and we observed a similar K_M for each dNTP with mutant and wild-type telomerase complexes (data not shown). Furthermore, we observed no increase in type-II processivity with increased dGTP concentrations with the mutant telomerase complexes, probably as a result of the low overall activity. In contrast, the wild-type enzyme displayed an expected dGTP-dependent increase in type-II processivity (*20*, *21*, *40*) (data not shown). In each case tested, the overall activity of the mutant telomerase complexes was below that of the wild type.

Activity of Chimeric Telomerase Mutants Varies with the Primer 3′ End. Tetrahymena telomerase can initiate extension of a primer that is aligned, on the basis of sequence complementarity, at any position from C49 to C43 (*27*, *28*). We examined if the primer alignment affects the efficiency of extension by the DNA-dependent telomerase mutants

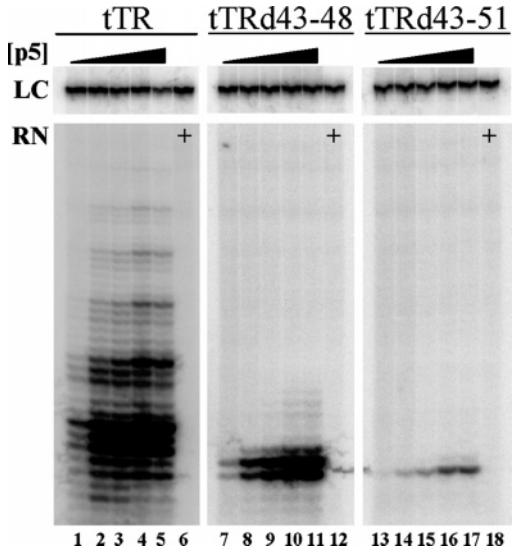

FIGURE 4: Primer concentration dependence of DNA-dependent and wild-type telomerase activity. Wild-type telomerase (lanes 1−6), telomerase reconstituted with tTRd43−48 (lanes 7−12), and telomerase reconstituted with tTRd43−51 (lanes 13−18) were assayed under standard assay conditions with increasing concentrations of primer p5 [$G(T_2G_4)_2TTGG$]: 0.1 μM (lanes 1, 7, and 13); 0.5 μM (lanes 2, 8, and 14); 2 μM (lanes 3, 6, 9, 12, 15, and 18); 10 μM (lanes 4, 10, and 16); and 20 μM (lanes 5, 11, and 17). A ^{32}P-labeled LC is indicated. Lanes labeled with RN are assays of samples pretreated with RNase.

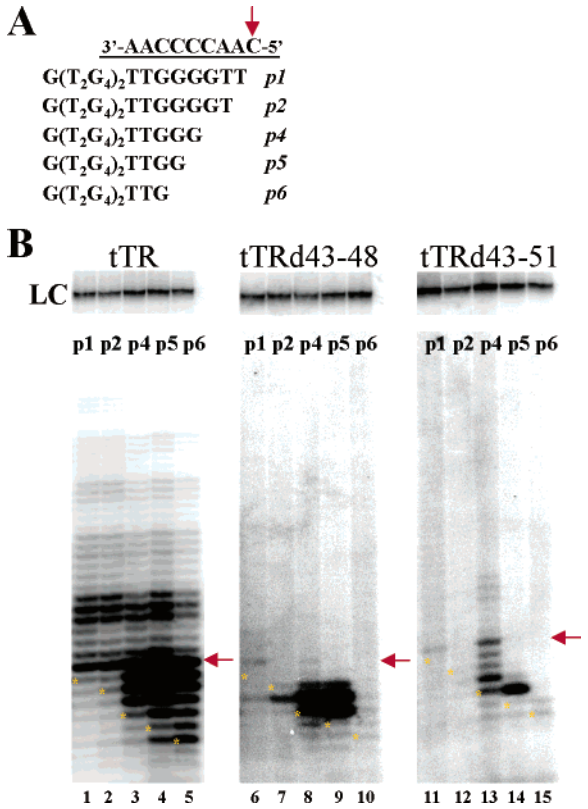

FIGURE 5: Affect of primer alignment on DNA-dependent and wild-type telomerase activity. (A) Schematic of the primer and template alignment of the five primers utilized in the primer extension assays. (B) Denaturing polyacrylamide gels of telomerase-catalyzed primer extension products using standard telomerase assay conditions and telomerase reconstituted with tTR (lanes 1−5), tTRd43−48 (lanes 6−10), and tTRd43−51 (lanes 11−15). An orange asterisk "*" denotes the length of the unextended primer for each primer used. The asterisk is placed at the left of side of the denoted band to avoid obstructing the band from view. The primer extended in each lane is indicated near the top of the gel. The red arrow indicates extension to the end of the template for all primers. A ^{32}P-labeled LC is indicated.

using a series of primers that align at five positions along the template (Figure 5A). We found, as expected, that wild-type telomerase is efficient at extending a DNA primer (lanes 1−5 of Figure 5B), with some specific primer alignments demonstrating increased activity (lanes 3−5). The activity of telomerase reconstituted with tTRd43−48 (lanes 6−10) was dramatically dependent upon primer alignment. Telomerase tTRd43−48 was most active extending primers p4 and p5 (lanes 8 and 9, respectively) for which the first and second nucleotides added are dGMP, respectively (note that the orange asterisks mark the length of unextended primers on the gel). With these primers, the addition of dTMP was generally inefficient. Notably, telomerase tTRd43−48 was incapable of extension to the end of the template with some primers (lanes 7, 9, and 10), while primers p1 and p4 were extended weakly to the template boundary (note the red arrow in lanes 6 and 8, respectively). tTRd43−48 telomerase exhibited a reduced overall activity with primers p1 (lane 6), p2 (lane 7), and p6 (lane 10) when compared to primers p4 and p5. This can be explained in part for p2 because the majority of the products from this primer result from cleavage of two nucleotides from the primer to template position C46 before addition of ^{32}P-dGMP, which is the most extensive product (lane 7, band below orange asterisk). Primer p1 was also at least partially excised by nuclease activity to template position C46 before extension (lane 6, band below orange asterisk). Primer p6 (lane 10), the poorest substrate of the six primers, can align with the template in two orientations: either fully extended to the template boundary or annealed to only the alignment domain. The inability to extend primer p6 suggests either that tTRd43−48 telomerase is incapable of adding a nucleotide across from the first template nucleotide, C48 (lane 10, light band above orange asterisk), following translocation, or that the fully extended DNA−DNA duplex is not translocated efficiently. Telomerase reconstituted with tTRd43−51 was unable to extend primer p2 (lane 12). Primers p4, p5, and p6 generated products that were shorter than the original primers, indicating that they were processed by nuclease activity to position C48 prior to extension (note bands equal to or shorter than the orange asterisk in lanes 13−15, respectively). For primer p5, the darkest band represents the addition of dGMP across template position C47 (lane 14, dark band above the orange asterisk), similar to the banding pattern seen for tTRd43−48 telomerase. Most important is the ability of tTRd43−51 telomerase to extend primer p4 to the end of the template (lane 13, red arrow) followed by primer realignment and a second round of extension, demonstrating the ability of telomerase to perform type-II processivity and therefore product translocation with a DNA template. Translocation efficiency was calculated as previously described (*39*, *40*) for primer p4, and we found that wild-type telomerase (translocation efficiency = 12%) was more efficient at primer translocation than tTRd43−48 telomerase (translocation efficiency = 5%).

Chain Terminators Reveal Decreased Nucleotide Addition Activity. To examine the effect of a DNA template on primer alignment, single nucleotide addition and nucleotide addition processivity (type-I processivity) in the absence of repeat addition processivity (type-II processivity), we utilized a

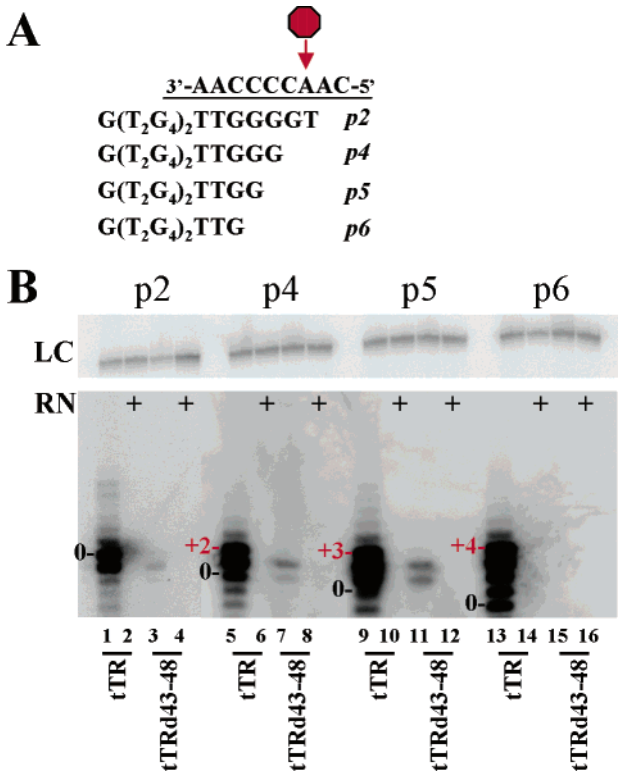

FIGURE 6: Dideoxythymidine primer extension stop assay confirms correct template−primer alignment and reduced nucleotide addition activity. (A) Template and primer sequences of the tested primers. The stop sign and arrow indicate the location of the first ddTMP (at A45), which terminates oligonucleotide primer extension. (B) tTERT assembled with tTR (lanes 1, 2, 5, 6, 9, 10, 13, and 14) or tTRd43−48 (3, 4, 7, 8, 11, 12, 15, and 16) was assayed under standard conditions, except that 100 μM ddTTP was substituted for dTTP. A black "0" indicates the original primer length, and red numbers indicate the position of ddTMP addition for each primer tested. A ^{32}P-labeled LC is indicated. Lanes labeled with RN are assays of samples pretreated with RNase.

dideoxythymidine triphosphate (ddTTP) stop assay modified from a previously described protocol (*41*). In the presence of ddTTP, *Tetrahymena* telomerase extends a primer to the position across from template residue A45 then adds a ddTMP residue, resulting in extension termination (Figure 6A, stop sign). With tTRd43−48 telomerase, primer extension did not proceed far enough to allow addition of ddTMP, suggesting that ddTMP addition is inefficient for this mutant (Figure 6B, lanes 3, 7, and 11). Because the banding pattern is not affected by ddTTP, we infer that primer alignment is the same in the DNA-dependent telomerase as in the wild type, although this is a tenuous assignment given the lack of robust activity. Of interest, primer p2, which should preclude the addition of ^{32}P-dGMP because ddTMP would be the first added nucleotide, did allow dGMP incorporation for both the wild-type and chimeric tTRs (lanes 1 and 3), suggesting that the primer is processed by a nuclease activity prior to extension. This previously observed nuclease activity (*37*) cleaved the primer at least back to position C46 before extension for both wild-type tTR and chimeric telomerase mutants (lanes 1 and 3 of Figure 6B, respectively; lane 7 of Figure 5B). The wild-type tTR telomerase had product bands shorter than or of equal length to the unextended primer for primers p4, p5, and p6 (lanes 5, 9, and 13, respectively), while the tTRd43−48 telomerase had a band equal in length to the primer for primer p4 (lane 7), suggesting that these primers were also processed by nuclease activity before extension. Primer p5 did not have products shorter than or equal in length to the primer when extended by tTRd43−48 telomerase (lane 11). Primer p6 was not extended by tTRd43−48 telomerase (lane 15). We did observe extension products past the A45 stop point with wild-type telomerase (lanes 1, 5, 9, and 13), which presumably resulted from contaminating dTTP present in the RRL.

Chimeric tTR Telomerases Efficiently Utilize an RNA Primer. In the previous experiments, we tested the ability of telomerase assembled with chimeric telomerase mutants to extend DNA primers. The next logical question to ask is whether the DNA-dependent telomerase mutants can extend an RNA primer, in effect reversing the polarity of the template−nascent product duplex from the native RNA−DNA to DNA−RNA. In this assay, dNTPs were used because telomerase is selective for dNTP substrates; thus, the product is a chimera. We tested two primers, a DNA primer, p5, and a chimeric primer containing ribonucleotides as the final four 3′ bases, p5rd (Figure 7A). This chimeric primer was used because an entirely RNA primer, p5r, was not extended by telomerase (data not shown), consistent with previous studies (*42*). Wild-type telomerase could efficiently extend p5 and p5rd (lanes 1 and 6 of Figure 7B, respectively) and exhibit type-II processivity (see green asterisks in lanes 1 and 6), but the overall activity was significantly decreased for p5rd (6.1% of p5 activity; lane 1 versus lane 6 of Figure 7B; Figure 7C). The tTRd43−48 telomerase was able to extend both primers (lanes 3 and 8), yielding an identical banding pattern (see blue and orange asterisks) but without type-II processivity. The activity with p5rd was decreased to 35% of the activity with p5 (lane 3 versus lane 8 of Figure 7B; Figure 7C). Similarly, tTRd43−51 telomerase extended p5rd with the same banding pattern as p5 (see blue asterisks in lanes 5 and 10) and with decreased activity, 27% of the activity with p5 (lane 5 versus lane 10 of Figure 7B; Figure 7C), and displayed no repeat addition processivity. To completely reverse the strand orientation of the template−nascent product duplex, telomerase would be required to accept NTPs as substrates. We attempted to use a tTERT mutant, Y623A, that displays decreased dNTP/NTP discrimination, to accomplish this result (*26*, *33*). However, the activity of the Y623A mutant was too low to recover activity with the chimeric templates (see the Supporting Information).

Template−Primer Thermal Stability. The reduced activity of telomerase when utilizing a DNA template could be a result of decreased stability of the template−nascent product duplex. In general, duplex thermal stability for identical sequences decreases in the order RNA−RNA > RNA−DNA > DNA−DNA. We used UV melting curves to determine the melting temperature for the four possible 9-base-pair duplexes formed by the template and primer (Table 1). Not surprisingly, the native duplex melting temperature of 41.0 °C is higher than the DNA−DNA melting temperature of 39.0 °C. This difference may contribute to the reduced activity level of the mutants. On the basis of this possibility, we examined the temperature dependence of telomerase-catalyzed primer extension. We found that a reduction in the assay temperature to 10 °C resulted in an equivalent decrease in the overall activity for both mutant and wild-type enzymes and an increase in the type-II processivity of the wild-type enzyme (see the Supporting Information). A

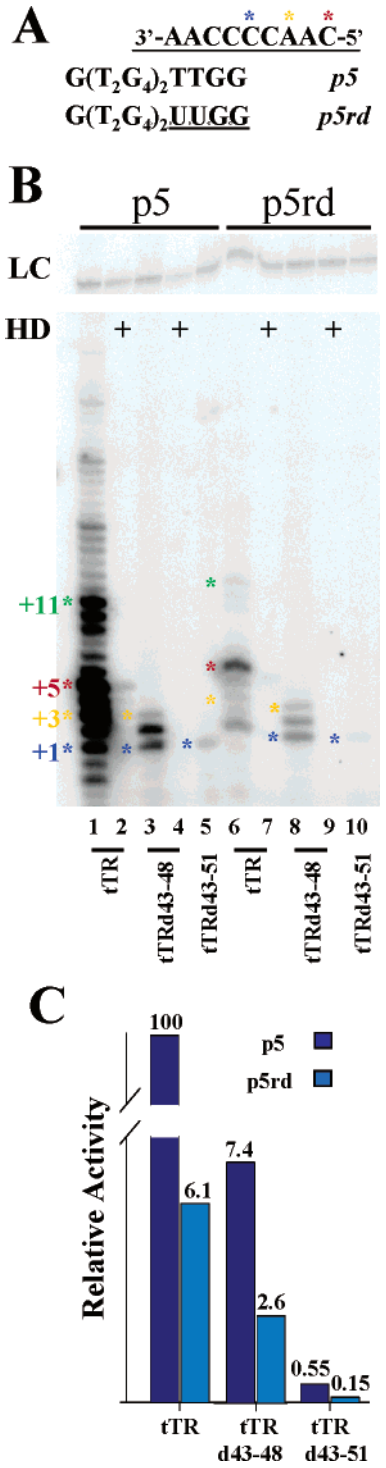

FIGURE 7: Extension of a chimeric RNA−DNA primer with telomerase assembled with chimeric tTR. (A) Alignment of template and primers tested in the assay. p5rd has NMPs (underlined) substituted for dNMPs at the last four 3′ positions. (B) Telomerase activity was assayed using standard conditions with 2 μM p5 primer (lanes 1−5) or 2 μM p5rd primer (lanes 6−10). Blue, orange, red, and green numbers and asterisks to the left of select lanes indicate the extension of the indicated primer by 1, 3, 5, and 11 nucleotides, respectively. Heat-denatured (HD) samples, 95 °C for 3 min prior to assay, served as a control for background activity instead of RNase, which would degrade the p5rd primer. A ^{32}P-labeled LC is indicated. (C) Quantification of reactions in B. Product intensities were normalized to the LC and to 100% activity, which was defined as the activity of wild-type telomerase with primer p5.

similar effect of temperature on processivity was recently reported by Aigner and Cech (*43*). Because decreasing the temperature did not rescue the activity of the mutant telomerase, it appears that decreased duplex stability does not explain the reduced ability of telomerase to function as a DNA-dependent polymerase.

Table 1: Duplex Melting Temperatures of Template−Primer Pairs[a]

template[b]	primer[c]	T_m[d] (°C)
RNA	DNA	41.0 ± 0.5[e]
DNA	DNA	39.0 ± 0.5
DNA	RNA	44.5 ± 0.5
RNA	RNA	60.0 ± 0.5

[a] Melting temperatures were determined from temperature-dependent UV absorbance measurements using 4 μM of each strand. [b] Template sequence: (5′-CAACCCCAA). [c] Primer sequence: (5′-TTGGGGTTG). [d] T_m was calculated from the first derivative of the melting curve at 260 nm. [e] Standard deviation from two separate determinations.

DISCUSSION

Telomerase is a unique RT, which uses a portion of its integral RNA subunit as a template for the extension of telomeric 3′ overhangs. In this paper, we described the synthesis and characterization of two mutants of *Tetrahymena* telomerase RNA that contain a deoxyribose backbone replacing the natural ribose backbone in the templating region. These chimeric tTR mutants allowed ribonucleoprotein (RNP) assemblage, enabling us to investigate the important features of the template−primer interactions, including template specificity and contributions of the structure of the template−nascent product duplex to activity. We showed, for the first time, that telomerase can utilize a DNA template. However, the activity of the DNA-dependent telomerase mutants was greatly reduced when compared to natural telomerase RT activity.

Telomerase exhibits three specific enzymatic activities: single nucleotide addition (*19*), telomere repeat addition (*19*), and nuclease activity (*37*). When telomerase functioned as a DNA-dependent DNA polymerase, we found that, while the activity levels were reduced (lane 1 versus lanes 3 and 4 of Figure 3), the hallmarks of wild-type telomerase activities were conserved. Telomerase is a RNP complex that requires its RNA subunit for both the template of DNA synthesis as well as enzymatic activity separate from its templating role. We found that telomerase assembled with either of the chimeric RNA subunits was sensitive to RNase (lanes 12 and 18 of Figure 4). Because the template in these chimeric molecules is DNA, the RNA sensitivity must come from the degradation of nontemplating portions of tTR. Because this occurred after telomerase was assembled, it offers further evidence that telomerase is a specialized and obligate RNP (*15*) and provides the first direct evidence that the RNA subunit is required for maintaining a functional active site even after the holoenzyme structure has been established.

During DNA synthesis, telomerase catalyzes two types of processivity. Type I is an extension of a primer to the template boundary, and type II is a repeat addition processivity, which requires translocation of the nascent DNA product to allow realigning after the template terminus has been reached (*38*, *39*). When telomerase utilized a DNA template, it was capable of limited primer extension (lanes 3 and 4 of Figure 3). Furthermore, dependent upon the primer used to initiate DNA synthesis, the DNA-dependent telom-

erase demonstrated both type-I (lanes 6, 8, 11, and 13 of Figure 5B) and type-II (lanes 10 and 11 of Figure 4; lane 13 of Figure 5B) processivity. These results demonstrate that the chimeric tTR-containing enzyme assembled correctly, properly aligned its template and primer in the active site, and could utilize a DNA template to extend a DNA primer. Thus, telomerase, similar to retroviral RTs, can utilize both DNA and RNA as a template. Telomerase is unique among RTs, however, in that its DNA-dependent activity is dramatically reduced compared to its RNA-dependent activity.

The low activity of the DNA-dependent telomerase mutants could be a result of several factors. The presence of a DNA template could alter the template−nascent primer helix geometry or reduce the thermal stability of the template−nascent primer duplex. We found that the thermal stability of the four possible template−primer duplexes does differ (see Table 1), but conducting assays at lower temperatures did not affect the activity of the DNA template-containing mutants (see the Supporting Information). However, the structure of the template−primer helix in the active site is likely to be affected by the presence of a DNA template. Two experimental observations support the hypothesis that telomerase is sensitive to the structure of the template−nascent product duplex. First, tTRd43−48 telomerase exhibits more robust type-I processivity than the tTRd43−51 telomerase (Figures 3, 4, and 5B), and second, both template mutants demonstrated a reduced ability to incorporate dTTP (Figures 3, 4, 5B, and 6B). The difference in activity of tTRd43−48 telomerase is likely due to the presence of RNA in the alignment region of this template mutant. These three additional RNA−DNA base pairs (A51−C49) could confer H- or A-form duplex character onto the primer annealed to the coding region of the template (C48−C43) (*47*). The less active mutant, tTRd43−51, contains DNA in the alignment region, which increases the possibility that the all DNA template−nascent primer duplex in the active site will tend toward a B-form structure (*47*). The decreased ability of the DNA-containing telomerase mutants to effectively add dTMP is consistent with the hypothesis that telomerase is sensitive to the helical structure contained in its active site, because T−A-rich DNA−DNA duplexes fail to form H- or A-form helices under normal conditions (*48*, *49*), whereas DNA−DNA duplexes high in G−C base pairs can easily be induced to form H- or A-form helices (*50*−*52*).

We made several efforts to force the template−nascent primer DNA−DNA duplex to assume a more A-form-like helix to recapitulate the natural helix shape and rescue primer extension activity (*53*). The addition of a dehydrating agent such as methanol, which has been used successfully to rescue activity in other polymerases by forcing the template−primer duplex to adopt an A-form helix (*54*), inhibited telomerase activity in general (data not shown). However, the telomerase containing a DNA template could utilize an RNA-containing primer with less reduction in activity than the wild-type enzyme when activity was compared to the extension of DNA primers (Figure 7B). There was a 16-fold decrease in the total activity of the wild-type telomerase when extending p5rd compared to a 2.8- and 3.7-fold decrease for tTRd43−48 and tTRd43−51 telomerase, respectively (Figure 7C). Perhaps the mutants are less affected by the use of an RNA primer than wild-type telomerase because the DNA−RNA duplex formed more closely resembles the duplex structure preferred by wild-type telomerase.

We conclude that *Tetrahymena* telomerase prefers an H- or A-form-like template−nascent product duplex. This assertion is consistent with published structural studies of RTs, RNA and DNA polymerases, and chimeric duplexes. The crystal structure of a covalently trapped catalytic complex of HIV RT (*55*) shows that approximately 5 base pairs of the DNA−DNA duplex in the active site are forced into an A-form helix, while the rest of the duplex is the B-form. This posits that HIV RT and possibly other RTs force the duplexes that reside in their active site into an A-form conformation regardless of the sugar backbone identity (*44*). This is not unique to RTs, because T7 RNA polymerase also shows evidence of forcing the A-form structure on its template−nascent product helix, and RNA polymerase can be converted to a DNA polymerase by the addition of a dehydrating reagent such as methanol (*54*). Also, crystal structures of *Bacillus stearothermophilus* DNA polymerase show an A-form DNA duplex in the active site, while the rest of the DNA is the B-form (*56*). Studies of poly(dG):poly(dC) duplexes have shown that these duplexes naturally form A-form structures in both crystal structures (*51*) and solution NMR structures (*50*). This may explain why telomerase demonstrates greater ability to utilize dGTP as a substrate when compared to dTTP when it functions with a DNA template.

The preference of telomerase for an H- or A-form helix in its active site may be a determinate of its fidelity and give rise to the apparent increase in the observed nuclease activity, which appears to precede nucleotide addition in many experiments (lanes 6−10 and 13−15 of Figure 5B; lanes 3 and 7 of Figure 6B). When an incorrect dNMP is added by the native enzyme, it likely causes a change in the local duplex helix geometry resulting in a nonoptimally positioned 3′-hydroxyl group, hindering nucleophilic attack on the incoming dNTP. In response, the enzyme could remove the added nucleotide as part of its proofreading mechanism. In our experimental system, the altered nucleotide is in the template and not in the primer; therefore, even correctly coded extension may be recognized as misincorporation and be removed, resulting in a futile cycle of addition−excision. DNA and RNA polymerases have proofreading domains that are separate from their active sites, whereas reverse transcriptases do not and are quite error prone with misincorporation rates of 10^{-1}−10^{-4} compared to 10^{-6}−10^{-9} for typical DNA polymerases (*44*, *45*). One study of human telomerase fidelity found error rates of 10^{-3} (*46*), which is similar to other RTs. Thus, one interpretation of our data is that the template−nascent DNA product duplex geometry within the telomerase active site plays a role in the fidelity of *Tetrahymena* telomerase, and this hypothesis is currently being investigated further.

Telomerase is a unique DNA polymerase because it utilizes its RNA subunit for both structural and enzymatic functions. The experiments described above demonstrate that telomerase can utilize a DNA template, although with decreased efficiency, to recapitulate the standard enzymatic functions of the wild-type enzyme including processive primer elongation and nuclease proofreading activity under standard conditions. Our results suggest that telomerase is sensitive to the structure of the nascent product−template

duplex. Importantly, the technology that we have described will allow further studies to more intimately probe the interactions between the telomerase template and the telomerase active site by placing any nucleotide analogue that can be incorporated by typical phosphoramidite chemistry into the telomerase active site.

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SUPPORTING INFORMATION AVAILABLE

Five figures illustrating the digestion of DNA template tTR with RQ1 DNase, a M-MLV RT assay with DNA template tTR used as a template for reverse transcription, a telomerase assay at reduced temperature, enzyme−primer K_M plots, and rNTP specificity of a previously described tTERT protein mutant (tTERT^{Y623A}) with chimeric tTRs. This material is available free of charge via the Internet at http://pubs.acs.org.

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