Effects of Nucleotide Analogues on *Euplotes aediculatus* Telomerase Processivity: Evidence for Product-Assisted Translocation†

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ABSTRACT: Telomerase is a unique ribonucleoprotein that reverse transcribes a defined region of its RNA subunit onto the ends of eukaryotic chromosomes. The product of telomerase, telomeric DNA, is typically a G-rich repeated sequence, $(TTTTGGGG)_n$ in the ciliate *Euplotes aediculatus* and $(TTAGGG)_n$ in humans. Telomerase can extend oligonucleotide primers in vitro in a processive fashion. We used dNTP analogues to study the structure−activity relationship between substrate nucleotides and processivity of telomerase from *E. aediculatus*. Several analogues, including 2′-deoxyuridine triphosphate (dUTP), 2′-deoxyinosine triphosphate (dITP), and 7-deaza-2′-deoxyguanosine triphosphate (7-deaza-dGTP), were good substrates for telomerase with K_m and V_{max} values near those of the natural substrates, dTTP and dGTP. However, telomerase processivity was affected with these substrates, decreasing in the order dUTP > 7-deaza-dGTP > dITP. Telomerase did not completely reverse transcribe the template when dITP was the substrate, and it efficiently extended a primer by the addition of two repeats when 7-deaza-dGTP and dUTP were utilized. When the same nucleotide analogues were incorporated into the primers, no effects were observed except in the case of a 3′-terminal deoxyinosine. The data support a model that includes the formation of an intramolecular secondary structure within the product DNA to facilitate translocation. The most likely structure is a G-G hairpin.

The ends of eukaryotic chromosomes are capped by a specialized DNA−protein complex called the telomere (*1*). Telomeres are critical for chromosome stability, and maintenance of telomeric DNA is essential for oncogenesis. Telomeric DNA consists of a simple repeated sequence, which differs from species to species but is generally G- and T-rich in the strand running 5′ to 3′ toward the chromosome end. Typical DNA polymerases are intrinsically incapable of reproducing the ends of linear DNA (*2*−*4*). To overcome this end-replication problem, most eukaryotes employ a specialized enzyme, telomerase, to synthesize telomeric DNA (*5*−*7*).

Telomerase is a ribonucleoprotein reverse transcriptase that synthesizes telomeric DNA using a portion of its RNA subunit as the template (Figure 1). In vitro, telomerase from most species can transcribe its template several times onto a single oligonucleotide primer (*8*−*10*). This type of processivity is unique to telomerase among reverse transcriptases. It requires unpairing of the extended DNA product from its RNA template and accurately realigning the 3′-end of the nascent DNA product with the template for further extension. During the translocation process, the telomerase active site must also be repositioned relative to the template. This translocation step is not dependent on GTP or ATP, typical intracellular energy sources. Understanding the mechanism of this precisely choreographed event is the impetus behind the research described here.

G-rich DNA is known to form several secondary structures including G-G hairpins and G-quadraplexes (*11*−*14*). This feature of G-rich DNA led to propositions that the structure of the DNA molecule produced by telomerase could regulate, either positively or negatively, its enzymatic activity (*15*−*18*). Zahler et al. showed that DNA in a G-quadruplex was not a substrate for telomerase from *Oxytricha nova* (*16*). In a separate study, Fletcher et al. showed that human telomerase activity was affected by monovalent cations (*17*). In the presence of potassium, there was an increase in the amount of telomerase products correlating with molecules that can form compact, i.e., G-quadruplex, structures. One conclusion that can be drawn from these studies is that G-quadruplex formation on the surface of telomerase inhibits further extension (*16*−*18*). On the other hand, mammalian telomerase is inhibited by 7-deaza-2′-deoxypurines in vitro, and the telomeric products are prematurely shortened (*19*−*21*). One interpretation of these results is that the ability to form secondary structure of the nascent DNA contributes positively to translocation, and the 7-deaza modification destabilizes that secondary structure. An intriguing model, suggested by Shippen and Blackburn, is that a G-G hairpin promotes translocation (*15*). The studies described here are consistent with that model.

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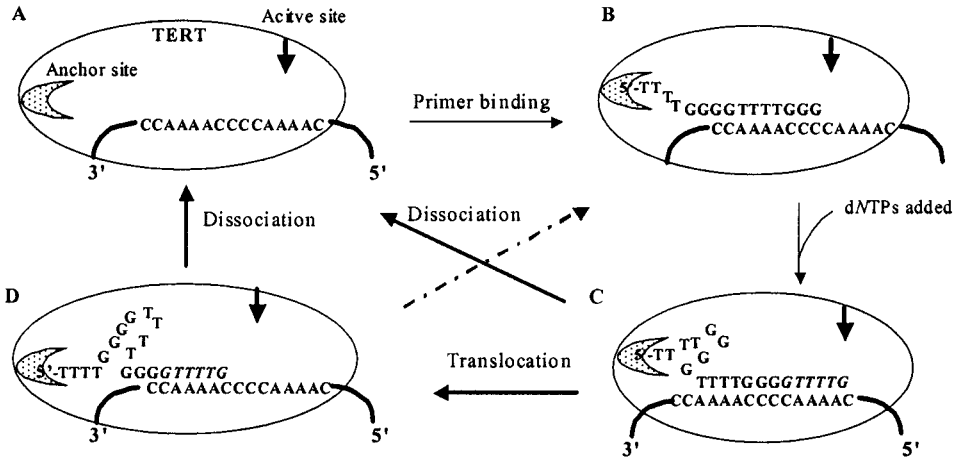

FIGURE 1: Telomerase-catalyzed polymerization of telomeric DNA. (A) Free telomerase with the sequence of the *E. aediculatus* RNA template sequence is given. (B) DNA (telomeric primer in vitro, 3′-end of chromosome in vivo) binds to telomerase. (C) Telomerase extends the telomeric primer by the addition of complementary dNTPs. When the end of the template is reached, the nascent DNA product can dissociate or translocate. (D) After translocation, the DNA primer is in position to be further extended, or it can dissociate. The order of translocation and dissociation is not known.

Telomerase from *E. aediculatus* was chosen for the current studies because all of its protein and RNA components are defined (*22*, *23*), and the conservation of the catalytic subunit, TERT,[1] throughout eukaryotes gives confidence that the results will be of general relevance (*6*). Particular attention was paid to the role of the structure of the nascent DNA product in translocation. We have analyzed dUTP, β,γ-methylene-dGTP (dGMPPCP), 7-deaza-dGTP, and dITP as substrates for telomerase from *Euplotes aediculatus*. The results with dUTP were further dissected by comparing the stability of an RNA·DNA duplex representing the extended product·RNA complex and an intramolecular G-quadruplex containing either dTTP or dUTP. The results indicate that the structure of the DNA product positively affects translocation for telomerase and are consistent with a DNA·RNA to G-hairpin transition providing part of the driving force for translocation.

MATERIALS AND METHODS

DNA Oligonucleotides. DNA oligonucleotides containing only unmodified bases were synthesized using standard phosphoramidite synthesis, and DNA oligonucleotides containing modified bases were purchased from Integrated DNA Technologies (Coralville, IA). All DNA oligonucleotides were purified by polyacrylamide gel electrophoresis. The RNA oligonucleotide was obtained from Dharmacon (Lafayette, CO), stored in the protected form, and deprotected following the protocol described by the manufacturer before use. DNA oligonucleotides were 5′-end labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol; NEN) and then purified using MicroSpin Sephadex G-25 TE columns. Sequences of the oligonucleotides used are given in the text.

Growth of E. aediculatus and Preparation of the Nuclear Extract. E. aediculatus was grown as described previously under nonsterile conditions with *Chlorogonium* as the food source (*24*). Cultures were grown in continuously aerated 5 gallon flasks. Nuclei were isolated by sucrose cushion centrifugation from 20−50 g cell pellets, and a nuclear extract was prepared by Dounce homogenization as previously described (*25*).

Preparation of Telomerase. Telomerase was partially purified by glycerol gradient centrifugation as described by Hammond and Cech (*26*). Fractions containing telomerase were identified by gel shift assays using 5′-^{32}P-labeled 5′-GGTTTTGGGGTTTTGCTTGACAG. Telomerase-containing fractions were pooled and dialyzed against reaction buffer. Preliminary experiments used this partially purified telomerase. For final experiments, telomerase was affinity-purified using a biotinylated oligonucleotide following the procedure described by Lingner and Cech (*23*), with several modifications: to 3−6 mL of partially purified telomerase from a glycerol gradient was added Nonident P-40 to 1% and 1 nmol of telomerase-specific biotinylated oligonucleotide/10 pmol of telomerase RNA. This solution was added to Ultralink- immobilized Neutravidin Plus (Pierce) beads (30 μL/pmol of telomerase RNA) and treated as described (*23*). Telomerase was released from the beads with the displacement oligonucleotide as described (*23*). Purified telomerase was dialyzed extensively versus reaction buffer [50 mM Tris-HCl (pH 7.5), 50 mM KGlu, 10 mM $MgCl_2$, 10% glycerol, and 1 mM DTT] to remove excess displacement oligonucleotide. This procedure afforded approximately 1000-fold purification from crude extract based on specific activity with 35% recovery based on quantitation of the RNA subunit.

Steady-State Kinetic Analysis of Telomerase. Assay mixtures (35 μL) contained reaction buffer, 10.5 fmol of affinity-purified telomerase, either telomeric primer P23 (50 nM, 5′-TTTTGGGGTTTTGGGGTTTTGGG) for analysis of deoxypurines or P24 (50 nM, 5′-TTTTGGGGTTTTGGGG-TTTTGGGG) for deoxypyrimidines, [α-^{32}P]dNTP [dTTP (10 μM) for analysis of deoxypurines or dGTP (6.5 μM) for analysis of deoxypyrimidines], and increasing concentrations of the unlabeled dNTP of interest. Reactions were incubated at 25 °C in reaction buffer, and 10 μL aliquots were removed at 3, 6, and 15 min and added to 50 μL of

[1] Abbreviations: 7-deaza-dGTP, 7-deaza-2′-deoxyguanosine triphosphate; dGMPPCP, β,γ-methylene-2′-deoxyguanosine triphosphate; dITP, 2′-deoxyinosine triphosphate; DTT, dithiothreitol; dUTP, 2′-deoxyuridine triphosphate; KGlu, potassium glutamate; TE, translocation efficiency; TERT, telomerase reverse transcriptase.

quench buffer[20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% SDS, 80 μg/mL proteinase K] containing 5′-^{32}P-labeled P20 (TTTTGGGGTTTTGGGGTTTT) as a loading control. Quenched reactions were incubated a further 45 min at 45 °C. The nucleic acid component was isolated by ethanol precipitation, resolved by electrophoresis on a denaturing 8% polyacrylamide gel, and imaged and quantified by phosphorimager analysis. A diluted sample of the stock [α-^{32}P]dNTP (10 and 100 fmol) was loaded onto the gel for the final 10 min to allow normalization of the phosphorimager intensity to the amount of radiolabeled nucleotide substrate. Initial velocities were determined by plotting femtomoles of nucleotide incorporated versus time and were linear for each assay over the time course of the experiment. Steady-state kinetic parameters were determined by plotting initial velocity versus concentration and fit to the equation $v_0 = V_{max}[S]/(K_m + [S])$.

Determination of IC$_{50}$. Assay mixtures (10 μL) contained reaction buffer, 3 fmol of affinity-purified telomerase, telomeric primer P23 (50 nM), [α-^{32}P]dNTP (dTTP for analysis of deoxypyrimidines or dGTP for analysis of deoxypurines), and increasing concentrations of inhibitor. Concentrations of [α-^{32}P]dNTP are given in the text. Reactions were incubated at 25 °C for 6 min and then terminated by the addition of quench buffer as described above. Products were isolated and quantified as described above, and the initial velocities were converted to percentages of total activity. The IC$_{50}$ was defined as the concentration of inhibitor that resulted in 50% inhibition by plotting percentage of activity versus inhibitor concentration and fit to the equation $100 = 100/(1 + [I]/IC_{50})$.

Single-Turnover Primer Extension Assay of Telomerase Activity. This assay was based on the primer extension assay described by Hammond and Cech (*9*). Purified telomerase (2.5 nM) was incubated with 5′-^{32}P-labeled telomeric primer (25 nM) for 5 min at 25 °C in reaction buffer. Aliquots (5 μL) of the reaction were mixed with an equal volume of telomerase reaction buffer containing dNTPs and unlabeled telomeric competitor primer (3 μM) and then were incubated for 20 min at 25 °C. Concentrations of the added nucleotides were varied in individual experiments. In some experiments, ddTTP was added to test for the register of the primer alignment. Reactions were stopped by the addition of 50 μL of quench buffer, incubated a further 45 min at 45 °C, and analyzed as described above. Translocation efficiency (TE) was determined using the equation

$$TE = 1 - [P_i/(P_i + P_{i+1} + ...)]$$

where P_i is the product band in question and P_{i+1} represents the product containing an additional telomeric repeat.

Thermal Denaturation Experiments. DNA·RNA duplex melting curves were carried out on a Cary double-beam UV−vis spectrophotometer equipped with a variable temperature controller. Absorbance values were recorded at 260 nm in 50 mM Tris-HCl (pH 7.5), 50 mM KGlu, 10 mM $MgCl_2$, 1 mM DTT, and 10% glycerol. Absorbance values were recorded once every minute and corrected against buffer blank. Duplexes were prepared by heating a 1:1 ratio of the RNA and test DNA oligomer at a concentration of 4.5 μM per strand to 95 °C for 5 min and cooling to room temperature at a rate of 2 °C/min. G-quadruplexes were prepared by heating a 7.5 μM solution of the test oligomer as described for the duplex-melting study, and melting curves were carried out as described for the duplex-melting study except that absorbance values were measured at 295 nm as described by Mergny et al. (*27*).

Table 1: Inhibition of Radionucleotide Incorporation by Telomerase Substrates

inhibitor	IC$_{50}$[a] (μM)	[α-^{32}P]dNTP (μM)	nonradioactive nucleotide (μM)
dTTP	10 ± 0.5[b]	dTTP (10)	dGTP (50)
dUTP	16 ± 1	dTTP (10)	dGTP (50)
dGTP	2.2 ± 0.1	dGTP (6.5)	dTTP (100)
dITP	3.5 ± 0.3	dGTP (6.5)	dTTP (100)
7-deaza-dGTP	3.0 ± 0.1	dGTP (6.5)	dTTP (100)
dGMPPCP	5000 ± 1000	dGTP (2.0)	dTTP (100)

[a] IC$_{50}$ values were determined as described in Materials and Methods. [b] Standard error of the values determined from the curve fit.

Table 2: Steady-State Kinetic Constants for Nucleotide Substrates[a]

substrate	K_m[b] (μM)	k_{cat}[c] (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
dTTP	4.0 ± 1.0[d]	10 ± 1.0	2.50
dUTP	16 ± 4.0	27 ± 1.0	1.70
dGTP	2.0 ± 0.4	27 ± 0.5	13.5
dITP	5.0 ± 1.0	1.7 ± 0.2	0.28
7-deaza-dGTP	19 ± 5.0	17 ± 1.2	0.89
dGMPPCP	1200 ± 160	0.17 ± 0.10	1.4×10^{-4}

[a] Assay conditions are given in the text. [b] Kinetic constants were determined as described in Materials and Methods. [c] Bulk k_{cat} based on total incorporation of radioactive nucleotide. [d] Standard error of the values determined from the curve fit.

RESULTS

The research described here was directed at defining the energy source for the translocation event that allows telomerase to catalyze the processive elongation of a telomeric primer. We used several substrate analogues to define structure−activity relationships for the dNTP substrates, oligonucleotide primer, and nascent DNA product.

Characterization of dTTP and dUTP as Telomerase Substrates. E. aediculatus telomerase was assayed with dGTP and either dUTP or dTTP as substrates to assess the effect of replacing the methyl group of thymine with hydrogen on telomerase activity. First, we determined the apparent affinity of dUTP and dTTP by assessing their ability to compete against incorporation of radiolabeled dTTP. We found that dUTP was as effective as unlabeled dTTP at inhibiting the incorporation of [α-^{32}P]dTTP (Table 1).

dUTP was then characterized as a substrate for telomerase. Time course experiments were run with varying concentrations of substrate (dUTP or dTTP) and a constant concentration of [α-^{32}P]dGTP. Telomerase produced a characteristic banding pattern of products with both substrates, although at low concentrations there was an increase in shorter products, presumably from increased stalling (Figure 2A,B). Interestingly, the first two major signals within the product-banding pattern had increased intensities when dUTP was the substrate (Figure 2B, bands +7 and +15). The observed K_m values for dTTP (4 μM) and dUTP (16 μM) are similar to those reported for endogenous *Tetrahymena* telomerase (*28*, *29*) and indicate that dUTP substituted for dTTP efficiently in this assay (Figure 2C,D and Table 2).

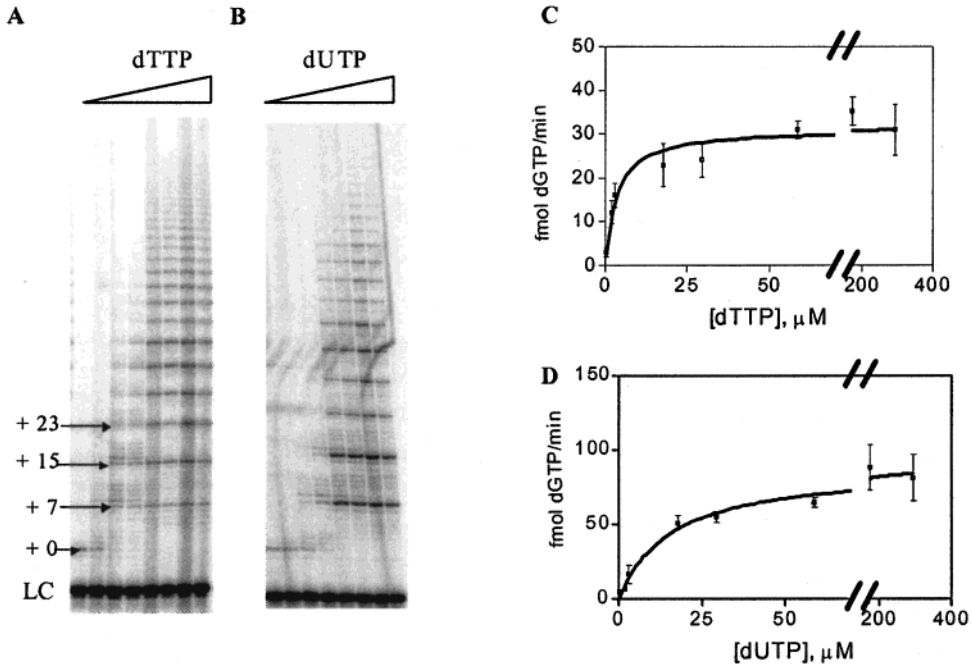

FIGURE 2: dUTP can substitute for dTTP in a telomerase assay. LC is a 20-nucleotide loading control. (A) Products of assay with increasing amounts of dTTP (0.29, 1.75, 2.94, 17.5, 29.4, 58.8, 175, 294 μM), [α-^{32}P]dGTP (6.5 μM), telomeric primer P24 (50 nM), and purified telomerase (0.3 nM). (B) Products from telomerase assay with increasing amounts of dUTP, [α-^{32}P]dGTP, telomeric primer P24, and purified telomerase (concentrations same as in part A). (C) Plot of the rate of [α-^{32}P]dGTP incorporation versus concentration of dTTP. (D) Plot of the rate of [α-^{32}P]dGTP incorporation versus concentration of dUTP. Curves were fit as described in the text.

The telomerase banding pattern can arise from distributive (nonprocessive) or processive elongation (*8*−*10*, *30*). In the processive mode, the banding pattern arises from a distribution of products generated during a single primer−enzyme interaction and is a direct result of pausing or increased dissociation during the catalytic cycle. The pausing presumably occurs prior to the translocation step, when the template 5′-end has been reached (*5*). In the distributive mode, primer is extended, released from the enzyme, and rebinds before it is extended again. To determine if the increased intensity of the first two bands in the pausing pattern when dUTP was the substrate resulted from an effect on distributive or processive elongation, a pulse−chase experiment was run in which radiolabeled primer was incubated with telomerase and then chased with unlabeled primer in the presence of substrates. This allows products generated from a single primer-binding event to be observed. Two concentrations of dGTP, 20 and 250 μM, were used since telomerase processivity can be affected by dGTP concentration *(9*, *10*, *31*).

In the pulse−chase experiment, telomerase produced shorter products overall with a noticeable increase in the first and second repeat products when dUTP was the substrate (Figure 3). The *E. aediculatus* specific dGTP concentration effect (each product being two nucleotides shorter with high dGTP) was not disrupted by the use of dUTP as a substrate. To test whether the identity of the first added nucleotide affected processivity, oligonucleotides ending in GGGG (P24) and TTTT (P20) were used (full sequence given in Figure 3). These require the initial addition of dTTP or of dGTP, respectively. As seen in Figure 3, the banding pattern was not dependent on the identity of the first added nucleotide.

When dUTP was the substrate and a dGTP concentration of 20 μM was used, the first translocation event occurred with high efficiency (TE = ~80%) while subsequent translocation events occurred with diminished efficiency (TE = ~50%). Under the same conditions with dTTP as the

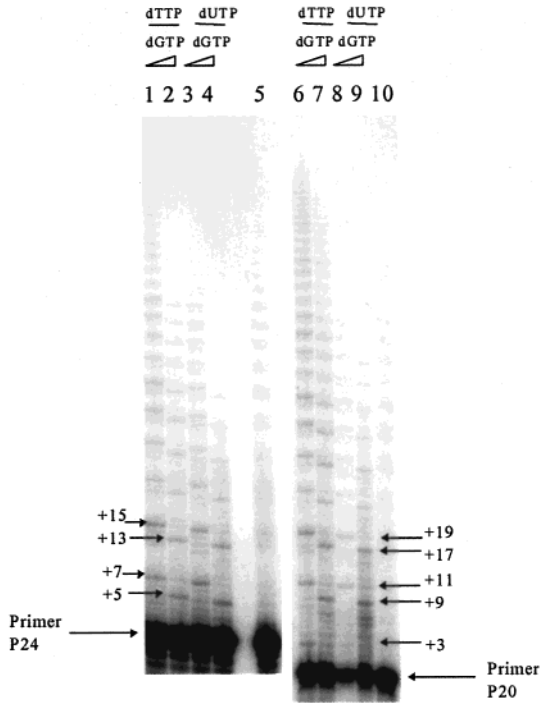

FIGURE 3: Telomerase processivity is different with dTTP or dUTP as the substrate. Lanes 1−5 contain products from a pulse−chase telomerase assay with 5′-^{32}P-labeled telomeric primer P20 (25 nM) and telomerase (2.5 nM). Lanes 6−10 contain products from a pulse−chase telomerase assay with 5′-^{32}P-labeled telomeric primer P24 (25 nM) and telomerase (2.5 nM). dTTP and dUTP concentrations were always 50 μM; dGTP was either 20 or 250 μM as indicated by the wedge. Lanes 5 and 10 are prechased controls with 3 μM unlabeled primer. P20 is 5′-TTTTGGGGTTTTGGGG-TTTT and P24 is 5′-TTTTGGGGTTTTGGGGTTTTGGGG.

substrate, all translocation events occurred with the same efficiency (TE = ~75%). The trend for dUTP, higher efficiency for the first translocation event compared to later events, was not dependent on either the dGTP or dUTP concentrations. It is apparent that telomerase is less processive when dUTP is the substrate as a result of the increased accumulation of the products representing addition of the first two complete repeats.

Characterization of Modified 2′-Deoxypurines as Telomerase Substrates. The concentration of dGTP affects processivity of human (*10*), *E. aediculatus* (*9*), and recombinant *Tetrahymena* (*31*) telomerase. To gain further understanding of this phenomenon, we analyzed a variety of dGTP analogues for their ability to affect processivity at varying concentrations. Two analogues, dITP and 7-deaza-dGTP, have changes in the structure of the base moiety that could affect substrate recognition and the structure of the DNA product. Another analogue, dGMPPCP, contains a nonscissile linkage between the β- and γ-phosphate linkage. This analogue was used specifically to test if cleavage of this linkage is required as an energy source for telomerase activity or processivity.

7-Deaza-dGTP and dITP were effective in preventing incorporation of [α-^{32}P]dGTP (Table 1), while dGMPPCP was a weaker binding inhibitor. At high concentrations of dGMPPCP a precipitate, presumably a complex of dGMPPCP and Mg^{2+}, was observed, making an accurate determination of the IC_{50} difficult. The ability of these analogues to replace dGTP as a substrate for telomerase was then determined. Both dITP and 7-deaza-dGTP were effective substrates, but their incorporation resulted in prematurely shortened products (Figure 4, Table 2). Telomerase only added one repeat before terminating extension when dITP was the substrate. With 7-deaza-dGTP, products representing two transcripts of the template were observed, indicating that translocation could occur at least once before product release. In contrast, dGMPPCP was a poor substitute for dGTP but did produce extension products of substantial length (data not shown). Both dITP and 7-deaza-dGTP showed substrate inhibition at high concentration, similar to dGTP.

To better examine telomerase processivity with the purine analogues, pulse−chase experiments were run with variable dGTP or dGTP analogue concentrations at a fixed dTTP concentration (Figure 5). As expected on the basis of the steady-state experiment, with dITP as the substrate, telomerase efficiently incorporated only one dIMP residue. This result indicates that telomerase is sensitive to the structure of the terminal residue of the DNA moiety and either pauses or dissociates from the nascent DNA product at an increased frequency when dIMP is added. Clearly, addition of dIMP inhibits the next nucleotide addition. A similar result with the addition of IMP has been observed with human RNA polymerase II (*32*).

With 7-deaza-dGTP, telomerase efficiently extended a primer by the addition of two repeats, but further extension was repressed (Figure 5). As with dGTP, there was a change in the banding pattern at high 7-deaza-dGTP concentration (*9*). In this case, instead of a shift to a different distinct pattern, an increase in pauses after the addition of each repeat was observed. Importantly, when 7-deaza-dGTP was the substrate and used at low concentrations, telomerase was efficient at the addition of the entire repeat sequence. This

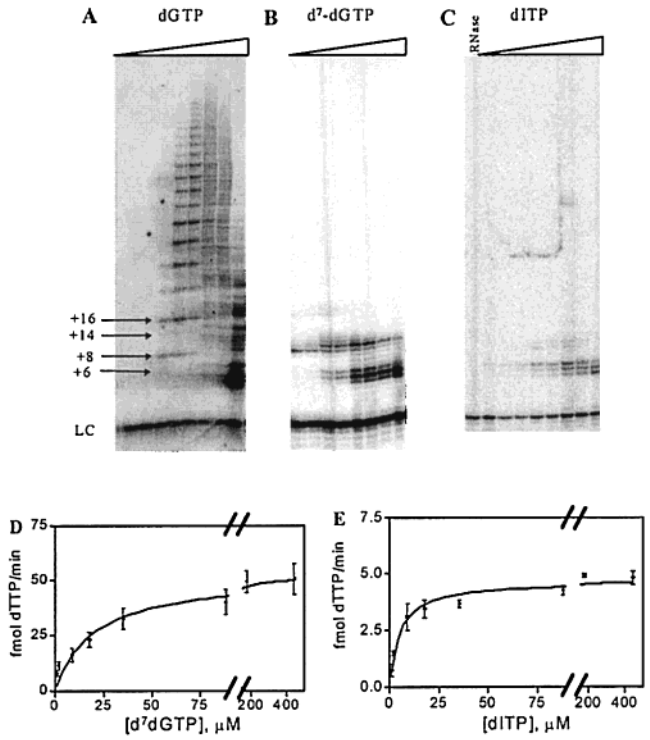

FIGURE 4: dITP, 7-deaza-dGTP, and dGMPPCP substitute for dGTP as telomerase substrates. Products from telomerase assays with increasing amounts of the following: (A) dGTP (0, 0.88, 1.76, 8.8, 17.6, 35.2, 88.0, 176 μM), [α-^{32}P]dTTP (10 μM), telomeric primer P23 (50 nM), and purified telomerase (0.3 nM); (B) 7-deaza-dGTP, [α-^{32}P]dTTP, telomeric primer P23, and purified telomerase (concentrations same as in part A); (C) dITP, [α-^{32}P]dTTP, telomeric primer P23, and purified telomerase (concentrations same as in part A). (D) Plot of the rate of [α-^{32}P]dTTP incorporation versus concentration of 7-deaza-dGTP. (E) Plot of the rate of [α-^{32}P]-dTTP incorporation versus concentration of dITP. Curves were fit as described in the text.

is indicated by the lack of increased products, when compared to the dGTP-dependent reaction, between the major pause sites (note the similarity between lanes 1 and 5 in panels A and B of Figure 5).

With dGMPPCP, telomerase produced longer DNA products than with the base-modified analogues, and the products always displayed the "low dGTP concentration" banding pattern (*9*). dGMPPCP also caused an increase of premature stops before addition of the first dGMP in the product, indicating that the nucleotide addition step is severely hampered by the presence of the methylene moiety. The fact that telomerase was processive, albeit with reduced activity, with dGMPPCP, convincingly demonstrates that cleavage of the β,γ-phosphate linkage is not required for translocation and, therefore, is not a potential energy source for processivity.

Effect of Primers Containing Modified Nucleotides. Because addition of nucleoside analogues to the nascent DNA product affected processivity, we hypothesized that the effect could be recapitulated by incorporating the modifications in the telomeric primer (see Figure 6 for a list of primers used). The ability of telomerase to extend the modified primers was tested at various primer concentrations by observing the incorporation of [α-^{32}P]dGTP. Each primer had an apparent K_m of 2−3 nM (data not shown). This suggests that the modified nucleotides do not dramatically affect the stability of the RNA·DNA duplex. A pulse−chase experiment was

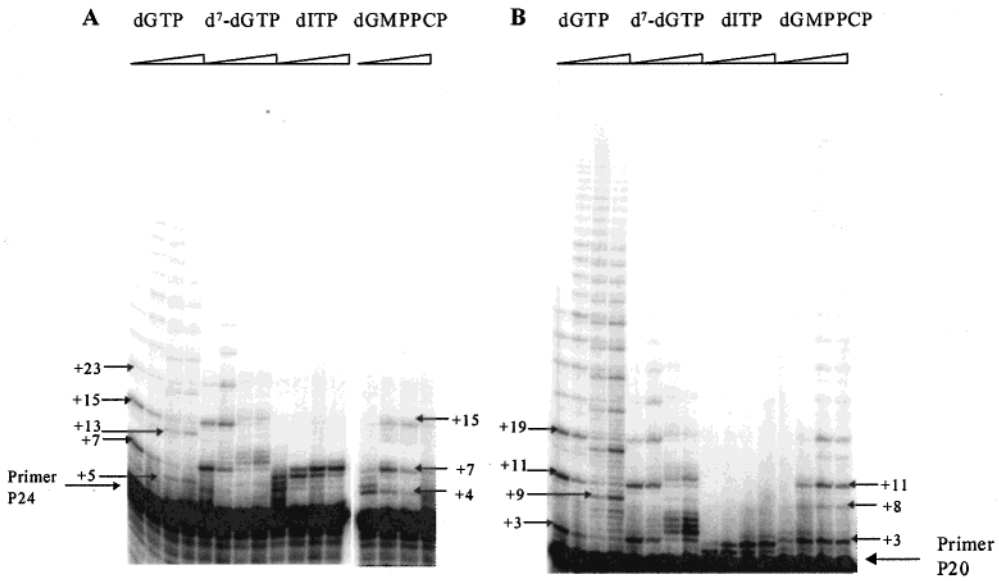

FIGURE 5: Telomerase processivity is different with dGTP, dITP, 7-deaza-dGTP, or dGMPPCP as the deoxypurine substrate. (A) Products from a pulse−chase telomerase assay with varying amounts of dGTP, dITP, 7-deaza-dGTP (10, 20, 100, 200 μM) or dGMPPCP (50, 250, 1000, 2000 μM), and dTTP (100 μM) with 5′-^{32}P-labeled telomeric primer P24 (25 nM) and purified telomerase (2.5 nM). (B) Products from a pulse−chase telomerase assay with varying amounts of dGTP, dITP, 7-deaza-dGTP (10, 20, 100, 200 μM) or dGMPPCP (50, 250, 1000, 2000 μM), and dTTP (100 μM) with 5′-^{32}P-labeled telomeric primer P20 (25 nM) and purified telomerase (2.5 nM).

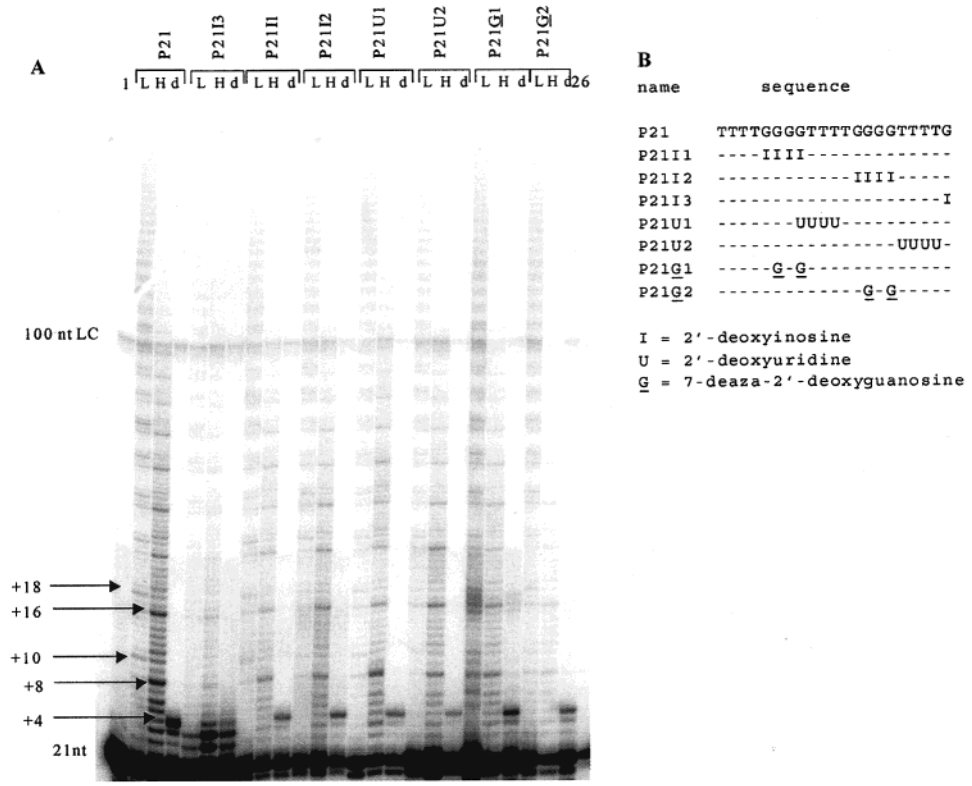

FIGURE 6: Effect of modifications in telomeric primers on telomerase processivity. LC is a 100-nucleotide loading control. (A) Products from a pulse−chase telomerase assay with dTTP (50 μM) and dGTP (10 μM, labeled L, or 250 μM, labeled H) or ddTTP (100 μM) and dGTP (100 μM, labeled d), 5′-^{32}P-labeled telomeric primers containing modified nucleotides (25 nM), and telomerase (2.5 nM). Lane 1 is a prechase control of P21 and lane 26 is an RNase control. (B) Primers used in the experiment.

utilized to determine the effect of the primer modifications on processivity (Figure 6). The percent of primer extended was similar (~10%) for each primer except 7-deaza-dGTP containing P21G2, for which <5% was extended.

Notably, the only modification that affected processivity was a 3′-terminal inosine, consistent with the observed inhibition of nucleotide addition after addition of dIMP described above. In this case, telomerase extended P21I3 in a processive reaction <1% of the time. The majority of the products resulted from termination after the addition of one or two nucleotides in the course of a single primer-binding event, consistent with the interaction between the RNA subunit and the 3′-terminus of the primer being important to the ability of telomerase to extend a primer. This contrasts

with results from a similar experiment with telomerase-active *Tetrahymena* extracts in which a primer containing a deoxyinosine at the 3′-end was extended as well as an unmodified primer (*33*). However, we found that the purity of the *E. aediculatus* enzyme preparation dictated the ability of telomerase to extend P21I3, suggesting that a contaminating nuclease may produce a natural end leading to proper extension. The result with the *Tetrahymena* telomerase can perhaps also be explained by the involvement of a nuclease activity, either inherent in telomerase or from a contaminating activity.

One possible cause of the differential activity of the modified primer, P21I3, relative to the parent primer could be incorrect alignment with the RNA template. To test for this possibility, we included the chain terminator ddTTP along with dGTP as substrates (Figure 6, lanes labeled d). All of the primers produced products of the expected +4 length with ddTTP except P21I3. The majority of the products with P21I3 continued to be +1 and +2 length. When compared to dTTP, ddTTP did not change the pausing pattern when p21I3 was the primer, consistent with P21I3 aligning properly with the template. The formation of short products in both cases was caused by inhibition of nucleotide addition after a dIMP residue.

Addition of Modified Nucleotide Substrates to Telomeric Primers Containing Modifications at the 3′-End. To test if the 3′-end of the telomeric primer interacts with newly added nucleotides, we examined the effect of combining analogues in the telomeric primer and nucleotide substrates. Figure 7 shows the gel of a pulse−chase experiment with several telomeric primers with modifications near the 3′-end and nucleoside triphosphate analogues. The effect of 7-deaza-dGTP and dUTP on processivity was the same with parent telomeric primer as with modified primers. Substrate identity did not affect the percent of primer extension in any of these experiments.

Thermal Stability of DNA·RNA Duplexes and G-Quadruplexes Containing dU. The incorporation of modified nucleotides into the nascent DNA product could affect the interaction between the RNA template and nascent DNA or the ability of the DNA to form a secondary structure such as a G-quadruplex or a G-hairpin. 7-Deazaguanine and deoxyinosine substitutions for guanine would be expected to preclude G-quadruplex formation, and this has been experimentally demonstrated (*12*, *16*, *33*, *34*). We determined the effect of substitution of dU for T in the DNA oligonucleotide of a DNA·RNA duplex representing the product and template of *E. aediculatus* telomerase (Figure 8A,B). Incorporation of dU into the DNA strand reduced the melting temperature of the duplex from 60 to 55 °C. We also examined the effect of substituting dU for T in a G-quadruplex with the *E. aediculatus* telomere sequence (Figure 8C,D). Again, a slightly destabilizing effect of dU substitution for T was observed.

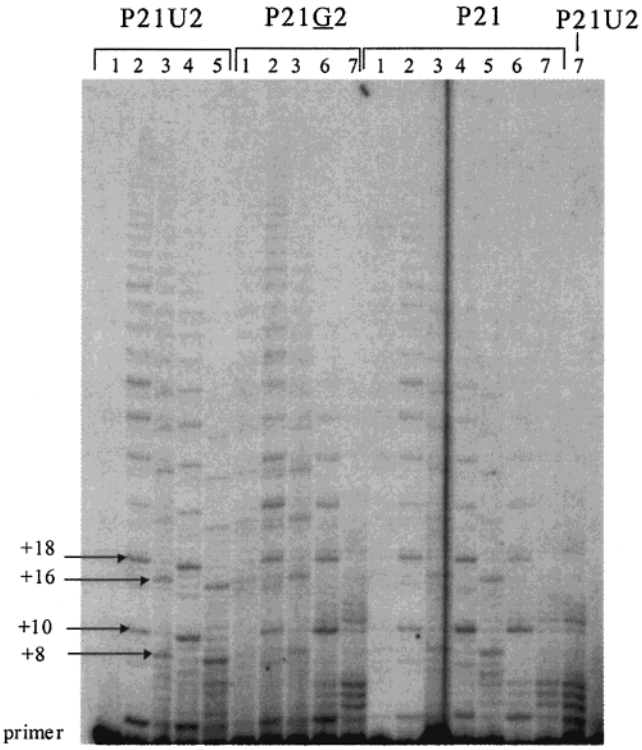

FIGURE 7: Combining modified nucleotides in telomeric primers and as substrates does not exacerbate the processivity defect. The polyacrylamide gel is of a pulse−chase telomerase assay. The identity of the ^{32}P-labeled primer is listed above the lanes. Primer (25 nM) and telomerase (2.5 nM) were the same in each experiment. Lanes: 1, prechased control with 3 μM unlabeled primer, dTTP (50 μM), and dGTP (250 μM); 2, dTTP (50 μM) and dGTP (10 μM); 3, dTTP (50 μM) and dGTP (250 μM); 4, dUTP (50 μM) and dGTP (10 μM); 5, dUTP (50 μM) and dGTP (250 μM); 6, dTTP (50 μM) and 7-deaza-dGTP (10 μM); 7, dTTP (50 μM) and 7-deaza-dGTP (250 μM).

DISCUSSION

Telomerase catalyzes the successive addition of telomeric repeats onto a DNA primer in vitro. This ability hinges on the efficient translocation of the nascent DNA product to realign the 3′-end of the DNA with the template. The experiments presented here were designed to examine the mechanism underlying the translocation event. Several nucleotide analogues were examined for their ability to replace the natural substrates, dGTP and dTTP. Some nucleotides were effective in allowing extension of a primer but resulted in decreased processivity. The most telling result was the lack of correlation between overall catalytic activity of telomerase with various substrate analogues and the processivity of the polymerization reaction. Instead, the strongest correlation was the relationship between processivity and the structure of the DNA product produced by the addition of the nucleotide analogues.

Effect of Nucleotide Analogues on Telomerase Activity. Each analogue tested had a unique effect on telomerase activity. The most extreme effect was produced by deoxyinosine. When dITP was utilized as the substrate or dIMP was present at the 3′-end of a telomeric primer, the addition of the next nucleotide was strongly inhibited, though incorporation of one dIMP residue was efficient. Similar effects have been observed for RNA polymerase II and were correlated to the proofreading mechanism of RNA Pol II (*32*). The effect presumably stems from the low stability of the I·C base pair (*35*).

A severe effect on activity was also observed with dGMPPCP, which was a very weak binding substrate and was incorporated very inefficiently. Despite this, telomerase was able to catalyze the complete synthesis of a telomeric repeat and translocate efficiently when dGMPPCP was the substrate. Obviously, a scissile linkage between the β- and

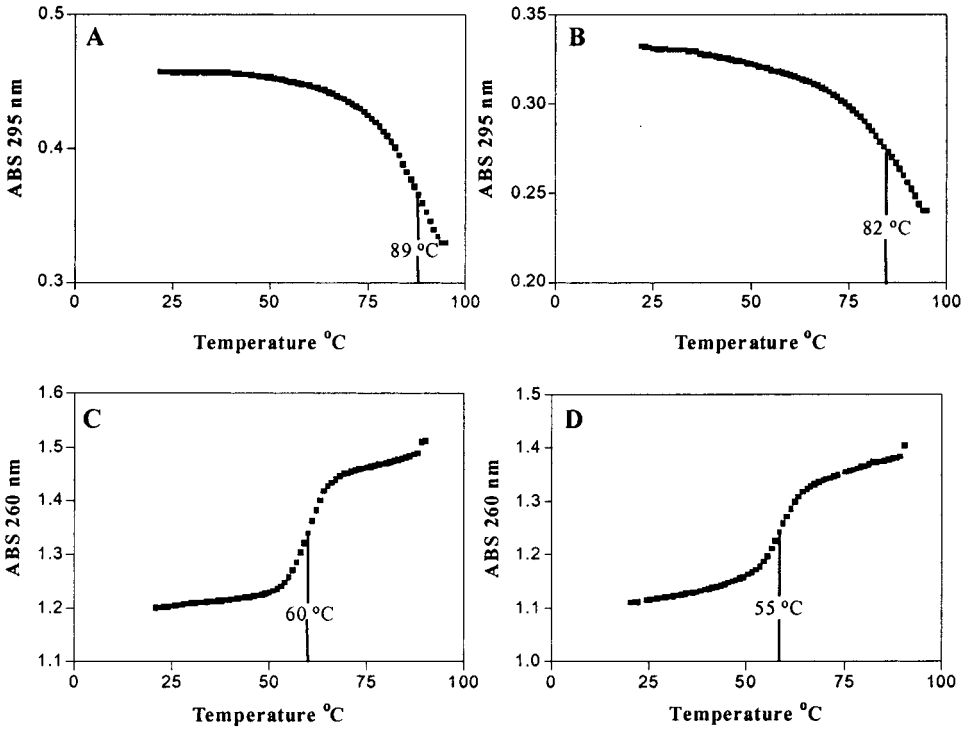

FIGURE 8: Substitution of deoxyuridine for thymidine into a G-quadruplex with the sequence 5′-$(GGGGTTTT)_3GGGG$ and into a DNA·RNA duplex with the sequence 5′-dGTTTTGGGGTTTTGG/3′-rCAAAACCCCAAAACC is slightly destabilizing. Melting curves of (A) G-quadruplex containing thymidine, (B) G-quadruplex containing deoxyuridine, (C) DNA·RNA duplex containing thymidine, and (D) DNA·RNA duplex containing deoxyuridine. T_ms were determined from the differential curve of the data.

γ-phosphates is not a requirement for translocation, appearing to eliminate the possibility that dGTP is an energy source for an intrinsic helicase-like activity. Our experiments do not rule out the possibility that hydrolysis of the β,γ-phosphate linkage of dTTP is an energy source, and this is currently under investigation.

When dGMPPCP was the substrate, telomerase paused more frequently at the addition of the first G. Since this additional pausing only occurred at one position in the template, it seems that affinity and/or activity of at least dGMPPCP varies depending on the position within the template. This is further supported by the finding that the length of the primer used to initiate synthesis did not dictate the position of the additional pause; it was always three nucleotides before the major pause. Although this was observed for a nonnatural substrate, it is likely that the same phenomenon, i.e., template-position-dependent activity, occurs for natural substrates but is obscured by the rapid polymerization involved in producing one transcript of the template.

A particularly interesting observation was the drastic change in processivity caused by dUTP and 7-deaza-dGTP. Both were efficient substrates with respect to their steady-state kinetic parameters; however, their incorporation caused telomerase to favor the production of shortened products. By comparison, other polymerases utilize 7-deaza-2′-deoxypurines and dUTP in place of dGTP and dTTP with varying efficiency but without changes in processivity (*36*-*42*). In the case of telomerase, the greatest effect of the modified nucleotides appears to be on the translocation step rather than the addition of individual nucleotides, since there is no increase in products intermediate in size between those that form the normal pausing pattern.

How the Structure of the Nascent DNA Could Affect Translocation. There are several reasons why the N7 of guanine and the methyl group of thymine could be required for efficient translocation. Interactions between the nascent DNA and the RNA template, interactions between the DNA and the protein active site or another subunit (p43 in the case of *E. aediculatus*), or intramolecular interactions of the DNA could be affected. We disfavor the possibility that the DNA·RNA duplex is disrupted because we observed only a modest effect from substituting dU for T on the thermal stability of a DNA·RNA duplex representing the fully extended DNA product base-paired to the RNA template. Others have reported that DNA duplexes are destabilized by dU incorporation (*43*, *44*). Elsewhere, it has been shown that replacing dG with 7-deaza-dGTP in alternating d(G−C) or d(C−G) oligomers also results in a 3 °C per d^7-dG residue decrease in melting temperature (*45*). The possibility that interactions between the protein and DNA product are affected has not been directly tested. Importantly, however, our observation that oligonucleotides containing d^7-dG and dU were efficient primers of telomerase activity suggests that the interactions between telomerase and nascent DNA are not greatly affected by these substitutions.

The third mentioned explanation for the observed nucleotide analogue effects involves an intramolecular interaction within the nascent DNA product. Several alternatives can be considered on the basis of known structures formed by G-rich DNA, including various G-quadruplex and G-G hairpin structures. We disfavor a model that includes a G-quadruplex structure as the intermediate in the processive telomerase reaction because the observed effect of dUTP on the thermal stability of a G-quadruplex was mild. However, there are several G-quadruplex topologies available for the

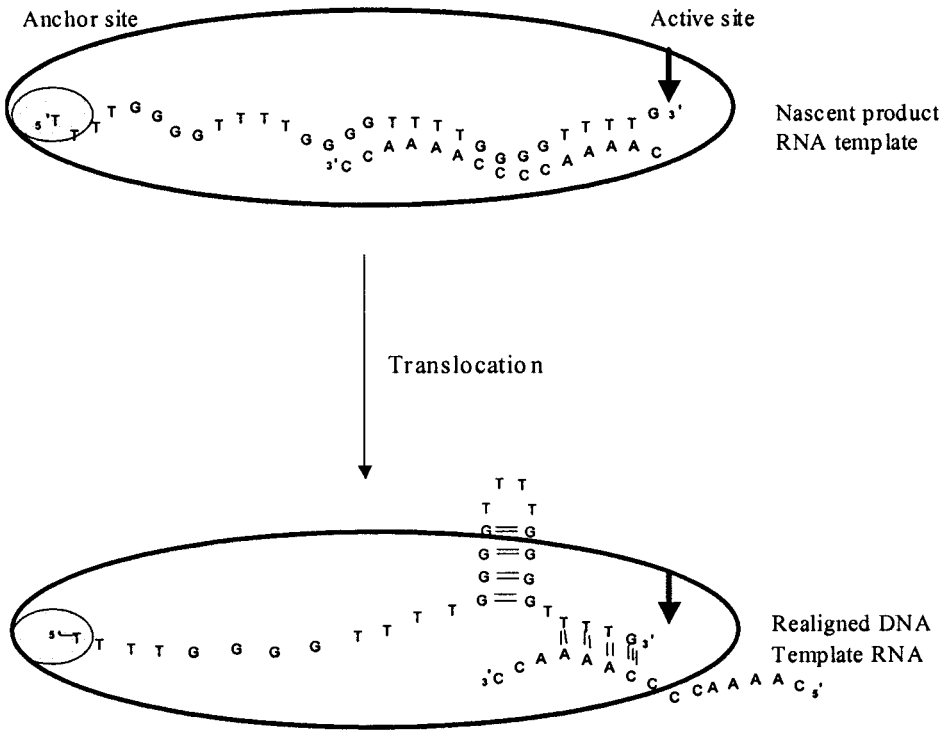

FIGURE 9: A model for G-G hairpin facilitating translocation. After the nascent DNA product reaches the end of the template, it must translocate to allow further extension. A G-G hairpin could facilitate this translocation step by replacing hydrogen bonds in the RNA·DNA duplex with intramolecular hydrogen bonds within the nascent DNA product.

E. aediculatus telomeric sequence. We cannot entirely rule out that one of these, not represented in our thermal stability studies, is involved in translocation. However, the melting temperature of G-quadruplexes, in general, is much higher than the temperature of the polymerization experiments, suggesting that G-quadruplex formation would be irreversible. This may explain why G-quadruplex formation appears to inhibit the processivity of human telomerase (*16*, *46*, *47*).

A G-G Hairpin Model of Translocation. One model that is consistent with the data is that a G-G hairpin, stabilized by Hoogsteen or reverse-Hoogsteen base pairing, facilitates translocation (Figure 9). Such a model was suggested after telomerase was recognized to be a ribonucleoprotein by Shippen and Blackburn (*15*). A G-G hairpin would have a low melting temperature, would form transiently, and would compensate for the energy required to melt the DNA-telomerase RNA duplex. Ogata and Miura invoked a similar hairpin structure to explain the ability of DNA polymerase from *Thermococcus litoralis* to expand a palindromic sequence (*48*). The *T. litoralis* DNA polymerase catalyzed the maximum processivity near the melting temperature, 70 °C, of the proposed hairpin intermediate. In that case, the hairpin was stabilized by Watson−Crick base pairing. The temperature effect on telomerase processivity is similar to that observed for *T. litoralis* DNA polymerase in that human telomerase (*49*, *50*) and *E. aediculatus* telomerase (*51*) exhibited the maximum processivity between 20 and 30 °C, similar to the expected melting temperature of a G-G hairpin. The human enzyme showed increased overall activity, measured by incorporation of radiolabeled nucleotides, as the temperature was increased to 37 °C, but produced shorter products (*50*). The melting temperature for a G-G hairpin has not been accurately determined. However, NMR experiments on the DNA sequence $G_4T_4G_4$ in Li^+ indicate that the melting temperature of the hairpin structure is between 25 and 35 °C (*52*).

Hydrogen bonds involving N7 of guanine are required for G-G hairpins, which are stabilized by Hoogsteen or reverse-Hoogsteen base pairing between two guanine residues. The necessity of the thymine methyl group is more tenuous. The methyl groups of thymines in the loops of the hairpin structure could interact with each other to produce stabilizing van der Waals interactions. That the substitution of dU for T causes a decrease in the melting temperature of a G-quadruplex is consistent with this conclusion, since G-quadruplex structures contain essentially two hairpins.

The G-G hairpin model is consistent with several observations reported for telomerase from other species. When the template of the *Tetrahymena* telomerase RNA was completely changed to a nonnatural sequence, telomerase was active but did not allow processive elongation (*53*). Other changes in the template of the *Tetrahymena* telomerase RNA caused a variety of effects including decreased processivity (*54*−*57*). These results can be explained in part by the G-G hairpin model, though the effects vary depending on the nature of the mutation. One intriguing possibility is that the position of the hairpin with respect to the active site is important in dictating efficient and accurate translocation. A series of circularly permuted telomerase template sequences tested by Autexier and Greider (*55*) were drastically affected in both translocation efficiency and precision in reconstitution assays in vitro, consistent with this model. Further, human telomerase, like the *E. aediculatus* enzyme, produces shorter products when 7-deaza-2′-deoxypurines are utilized (*19*).

The ability to produce several copies of telomeric sequence during the course of a single primer−template interaction seems to be a property of telomeric DNA in particular and

not necessarily telomerase. Nozawa et al. (*58*) have shown that DNA polymerase α-primase, the Klenow fragment of *E. coli* DNA polymerase I, and HIV reverse transcriptase can expand a telomeric sequence $(TTAGGG)_n$, in vitro, beyond the length of the supplied template $(CCCATT)_n$. Further, this polymerization reaction was not a simple slippage reaction, in which case a continuous ladder of products might be expected, but, instead, a human telomerase-like translocation reaction, which produced a ladder of products separated by six nucleotides. This is exactly the length of the human telomeric repeat sequence found in the template. It is likely that a G-G hairpin is responsible for these observations.

The hairpin model for translocation could be important not only because it helps to explain translocation by telomerase but also because it predicts that telomerase will translocate in a fashion that maintains the sequence integrity of the repeated DNA sequence. Hairpin-driven translocation would force the nascent DNA to reposition in the correct register for accurate telomeric DNA synthesis. This is because for the human, *Tetrahymena*, and *E. aediculatus* telomerase reactions, the hairpin formed after successive repeat additions contains exactly two repeats. One and a half repeats form the actual hairpin, while the other half is available to align with the template. The 5′ set of dG residues in the hairpin originates from the 3′-end of the previously formed hairpin. Thus, the original "inchworm" model of Shippen and Blackburn (*15*) explains not just the processive activity of telomerase but possibly the fidelity of repeat addition to telomeres as well. Blackburn and co-workers described one example that is consistent with this conclusion (*56*, *59*). A *Tetrahymena* template mutant that coded for the sequence T_2G_5 instead of T_2G_4 showed a loss of fidelity with products of the sequence T_2G_6, T_2G_7, and T_2G_8 forming together with the expected T_2G_5. Perhaps translocation was no longer facilitated by a G-G hairpin and instead a slippage mechanism dominated, giving rise to misincorporation of extra dGMP residues.

CONCLUSION

The structure−activity relationship for the dNTP substrates of telomerase reported here indicates that telomerase is sensitive to the structure of the nascent DNA product. The data are consistent with the formation of a G-G hairpin as a thermodynamic driving force for translocation of the nascent DNA product to allow continued extension and therefore processive elongation. The model also explains the high fidelity of processive repeat addition catalyzed by telomerase from various organisms.

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