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Sequence-Specific DNA Primer Effects on Telomerase Polymerization Activity

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The ribonucleoprotein enzyme telomerase synthesizes one strand of telomeric DNA by copying a template sequence within the RNA moiety of the enzyme. Kinetic studies of this polymerization reaction were used to analyze the mechanism and properties of the telomerase from *Tetrahymena thermophila*. This enzyme synthesizes TTGGGG repeats, the telomeric DNA sequence of this species, by elongating a DNA primer whose 3′ end base pairs with the template-forming domain of the RNA. The enzyme was found to act nonprocessively with short (10- to 12-nucleotide) primers but to become processive as TTGGGG repeats were added. Variation of the 5′ sequences of short primers with a common 3′ end identified sequence-specific effects which are distinct from those involving base pairing of the 3′ end of the primer with the RNA template and which can markedly induce enzyme activity by increasing the catalytic rate of the telomerase polymerization reaction. These results identify an additional mechanistic basis for telomere and DNA end recognition by telomerase in vivo.

Telomeres are the structures at the ends of eukaryotic chromosomes and are essential for chromosome stability and long-term maintenance (1, 4, 21). Telomeric DNA typically consists of repeated simple sequences specified by the enzyme telomerase. Telomerase is a ribonucleoprotein enzyme which synthesizes one strand of the telomeric repeats by copying a template sequence in the RNA moiety of telomerase (8, 9, 17, 19). The mechanism of synthesis of the G-strand telomeric DNA by telomerase from the ciliate *Tetrahymena thermophila* shown schematically in Fig. 1 is supported by previous work (reviewed in reference 2). Copying of the template sequence 5′-CAACCCCAA-3′ results in the synthesis of one strand of telomeric DNA with the repeating hexameric sequence TTGGGG. Previous work has shown that with a primer whose 3′ end is complementary to the template, the next residue added is defined by the 3′-end sequence of the primer (8, 9). The overall reaction consists of several steps. Binding of primer and deoxynucleoside triphosphate (dNTP) to telomerase and extension on the RNA template produce T_2G_4 repeats. An implied step in processive polymerization of multiple T_2G_4 repeats (3, 6) is the dissociation of the growing 3′ end of the product from the template and its translocation to the partially repeated template sequence at the 3′ end of the template-forming domain (Fig. 1).

Previous work has shown that telomerase exhibits specificity for the sequence of the DNA primer. Such specificity might be expected to have implications for the in vivo recognition of telomeres by telomerase and also for the efficiency of healing of nontelomeric, broken chromosome ends by telomerase. However, no detailed kinetic study of the action of telomerase has been reported previously, and the mechanistic basis for primer specificity, beyond that determined by the complementarity of its 3′ end to the RNA template, has been poorly understood.

In previous studies in which several different DNA oligonucleotides were tested as primers for telomerase activities from *Tetrahymena*, *Oxytricha*, *Euplotes*, and human cells (3, 7–10, 13, 16, 17, 20; unpublished data), various features of the primer were found to affect its efficiency of utilization. However, variation of both the sequences and lengths of the primers made it difficult to assess which features were critical in causing their differential priming efficiencies. In addition, previous studies left unclear the roles in primer efficiency played by primer-binding affinity as opposed to other aspects of the kinetics of the telomerase reaction.

In this study we used kinetic analyses to study telomerase-primer interactions. Specifically, we compared the kinetic parameters of primers of constant length whose sequences shared the same degree of template hybridization at the 3′ end but varied at the 5′ end. We showed that the 5′-end sequence of the primer can dramatically affect the catalytic rate constant of the telomerase polymerization reaction. These results provide the first evidence that sequence-specific interactions between the primer and telomerase, distinct from template hybridization, can have an inductive effect on the active site of the enzyme.

MATERIALS AND METHODS

Telomerase enzyme preparations. Preparations were done essentially as described previously (8) with the modifications described below. Between column chromatography steps during the purification, fractions were stored frozen at −70°C. S100 extracts from mated *T. thermophila* cells were fractionated on Sephacryl S-500 HR (Pharmacia) in TMG buffer (10 mM Tris-HCl [pH 8.0], 1 mM $MgCl_2$, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride). Active fractions were pooled and loaded onto a heparin-agarose (Bio-Rad) column at a ratio of 3 ml of extract to 1 ml of resin. Telomerase activity was eluted with 100 mM sodium acetate in TMG buffer. Active fractions were loaded onto a DEAE-agarose (Bio-Rad) column in 100 mM sodium acetate-TMG. A 200 mM sodium acetate step preceded elution of telomerase activity with 300 mM sodium acetate. These fractions were brought to 500 mM sodium acetate and loaded onto an octyl-Sepharose (Pharmacia) column. The column was washed with 3 to 4 column volumes of TMG, and telomerase activity was eluted with TMG containing 1% Triton X-100.

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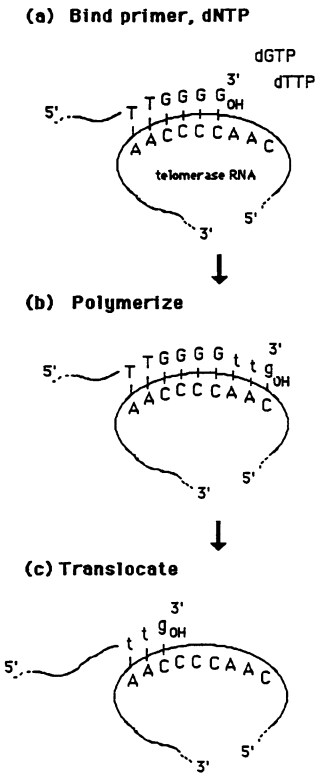

FIG. 1. Mechanism of telomerase activity. The template-forming domain of the telomerase RNA moiety is shown. (a) A primer ending in -TTGGGG binds by hybridizing with the template domain of the telomerase RNA. (b) Templated polymerization occurs, elongating the 3′ end of the primer. (c) When the 5′ end of the template is reached, translocation of the product to the 3′ end of the template-forming sequence allows synthesis of T_2G_4 repeats.

Fractions were collected in tubes which contained ultrapure acetylated bovine serum albumin (BSA; United States Biochemicals) to a final concentration of 0.5 mg/ml to minimize loss of activity on freezing at −70°C. All experiments described in this paper were done with octyl-Sepharose-purified material (unless otherwise noted).

The fold purification of telomerase activity in octyl-Sepharose fractions was difficult to determine because of interference of Triton X-100 with protein assay reagents. Heparin-agarose-purified material is about 60-fold purified relative to the S100 fraction (6), and octyl-Sepharose-purified material was estimated to be purified a further 20-fold.

Determination of contaminating activities in telomerase preparations. (i) 3′-5′ exonuclease activity. Fractions were tested for 3′-5′ exonuclease and endonuclease activities on telomeric oligonucleotides labeled at the 3′ end with a ^{32}P residue. The oligonucleotide $T_2G_4T_2G_3$ was 3′ end labeled with [α-^{32}P]dNTP by terminal deoxynucleotidyltransferase. Products were separated by gel electrophoresis, and products resulting from the addition of one or two nucleotide residues were excised and eluted from the gel and ethanol precipitated with glycogen. Telomerase fractions (both heparin-agarose and octyl-Sepharose purified) were incubated for various times at 30°C under telomerase assay conditions (see below) with the 3′-end-labeled substrates present in low concentrations. Loss of radioactivity was assayed by filter binding (7). After 5 min of incubation, 80 to 90% of the starting radioactivity remained for octyl-Sepharose samples, compared with 50% for heparin-agarose samples. Octyl-Sepharose material in this experiment, per volume of enzyme, was approximately twice as active in telomerase activity as was the heparin-agarose material (judged by the intensity of products on autoradiogram). The nuclease activity was not RNase sensitive and did not show telomerase primer specificity; it is therefore unlikely to be an intrinsic activity of the telomerase enzyme. Telomerase kinetic experiments were done with a large excess of unlabeled oligonucleotide primer, and no evidence for primer degradation was found, indicating that this degree of contaminating nuclease was unlikely to affect the overall results.

(ii) Other DNA polymerase activity. Telomerase fractions were tested for DNA polymerase activity by using 1 μg of poly(dA-dT)$_{16}$ and 1.25 μM [α-^{32}P]dTTP. Heparin-agarose- or octyl-Sepharose-purified telomerase fractions or 5 and 0.5 U of Klenow fragment DNA polymerase (New England BioLabs) were incubated at 30°C for 30 min in telomerase assay buffer.

Filter-binding assays showed that heparin-agarose-purified and octyl-Sepharose-purified fractions contained <1% and <0.1%, respectively, of the polymerase activity present in the positive Klenow fragment control. No products were detected by polyacrylamide gel electrophoresis (PAGE) for the heparin-agarose and octyl-Sepharose samples.

Telomerase reactions. Telomerase reactions were done essentially as previously described (8) with the modifications described below. All reaction mixtures contained a 0.5 volume of telomerase in storage buffer (1% Triton X-100 in TMG with 0.5 mg of BSA per ml). Assay buffer was added so that final reaction mixtures contained an additional 50 mM Tris (pH 8.0 at 25°C), 1 mM spermidine, and 1 mM dithiothreitol.

Two types of telomerase reactions were done, one in which [α-^{32}P]dGTP and dTTP were present in the reaction mix (G+T assay) and the other in which only [α-^{32}P]dTTP was present (T-only assay). For the G+T assay, final reaction concentrations of dNTPs were 1.25 μM [α-^{32}P]dGTP (400 Ci/mmol) and 100 μM cold dTTP. For the T-only assay, a final reaction concentration of 1.25 μM [α-^{32}P]dTTP (400 Ci/mmol) was used (in some reactions, additional cold dTTP was added to 10 μM).

Unless otherwise stated, all telomerase reaction mixtures contained DNA oligonucleotide primer and the reaction components described above.

Reactions were terminated with 10 volumes of stop mix (10 mM Tris [pH 7.5], 1 mM EDTA), extracted once with phenol-chloroform (50:50, vol/vol), and precipitated with an equal volume of 5 M ammonium acetate, containing 20 μg of glycogen, and 700 μl of ice-cold ethanol for at least 15 min. Samples were run on 7 M urea–polyacrylamide sequencing gels (0.6× TBE) varying from 8 to 15% polyacrylamide. Dried gels were exposed to conventional autoradiographic film and/or a phosphor imaging screen for quantitation.

Primer titration assays. For each reaction, a tube containing the oligonucleotide in 3 μl of assay buffer was heated at 90°C for 1 min (to melt out any intermolecular forms which G-rich DNA can form [13, 18]) and equilibrated at 30°C for 10 min. A 7-μl aliquot of enzyme in assay buffer was prewarmed at 30°C for 1 min, added to the oligonucleotide tube, and incubated for 5 min at 30°C to allow enzyme-primer complexes to form. The telomerase reaction was initiated by the addition of 2 μl of the dNTP solution. Reactions were stopped after 5 min, and the products were processed as above.

Reaction products were separated by PAGE and analyzed

by PhosphorImager. For 12-nucleotide (nt) primers at 1.25 μM dTTP and 10-nt primers at 10 μM dTTP in the T-only assay, the t2 band was a significant proportion of the total reaction product. The rate of t1 product formation was therefore calculated as t1 + 0.5t2, based on the reasoning that half of the ^{32}P label in the t2 band came from depleting the t1 band into t2. For the 10-nt primers at 1.25 μM dTTP, the proportion of t2 was at most 20% of that in t1. For these primers, kinetic calculations and comparisons were based on the amount of radioactivity incorporated into the t1 band alone. Least-squares fitting lines of the data in Eadie-Hofstee plots yielded the V_{max} (y-intercept) and K_m (negative-slope) values. t2/t1 ratios were calculated by dividing the label in t2 by that in t1 at each primer concentration, without correcting for the presence of two ^{32}P labels in t2.

T-only time course to determine biphasic kinetics. Batch solutions of oligonucleotide, enzyme, and dTTP were incubated and mixed in the order described above for the primer titration assays. Oligonucleotide was present at saturating concentration, and enzyme mixes (for 32, 16, or 8 μl of octyl-Sepharose telomerase fraction) were made by 1:1 serial dilutions into TMG containing 1% Triton X-100 and 0.5 mg of BSA per ml. dTTP was added to 10 μM and a specific activity of 50 Ci/mmol. Aliquots (12 μl) of each initial 64-μl reaction mixture were removed into stop mix at 0.5-min intervals. Samples were analyzed by gel electrophoresis and phosphor imaging quantitation as described above.

To calculate the concentration of active sites, we excised bands from the dried gel and used scintillation counting to determine the extent of radioactivity incorporated. The number of moles of product formed from PhosphorImager-generated quantitation of bands t1 + 0.5t2 was calculated from the conversion factor 2.2×10^6 dpm or cpm/μCi and the specific activity of [α-^{32}P]dTTP (50 Ci/mmol). The slope of product formed plotted against the amount (volume) of telomerase fraction gives a minimal estimate of telomerase active-site concentration. This number was 3×10^{-3} and 5×10^{-3} fmol/μl in two experiments with the same enzyme preparation and 6×10^{-3} and 7×10^{-3} fmol/μl in two later experiments with a different enzyme preparation.

Determination of K_m for dNTPs. For determination of the K_m for dGTP, a batch solution of telomerase and dNTPs in telomerase assay buffer was prepared to the final concentrations described above for the G+T reactions. Tubes were prepared which contained 1 μl of the oligonucleotide primer $(T_2G_4)_2$ at a final concentration of 100 nM and 4 μl of cold dGTP to final concentrations of 1, 5, 10, 50 and 100 μM. Reactions were initiated by adding 15 μl of the telomerase and dNTP batch solution to each tube of oligonucleotide plus cold dGTP. After incubation at 30°C for 30 min, reactions were spotted onto DE-81 filter papers, washed, and counted by liquid scintillation. For determination of the K_m for dTTP, the batch solution contained 1.25 μM [α-^{32}P]dTTP, 2 μM dGTP, and heparin-agarose-purified rather than octyl-Sepharose-purified material. Tubes containing 1 μl of the primer $(T_2G_4)_2$ at a final concentration of 1 μM were prepared. Each tube of oligonucleotide was heated at 90°C for 1 min and equilibrated at 30°C for 10 min, after which 15 μl of the telomerase plus dNTP batch solution and 4 μl of cold dTTP to final concentrations of 1, 10, 50, and 100 μM were added. The counts obtained at each concentration of cold dNTP were multiplied by the fold dilution of the [^{32}P]dNTP. Eadie-Hofstee plots were generated, and the K_m values for dGTP and dTTP were determined. The K_m values for dGTP and dTTP were 1 and 5 μM, respectively.

RESULTS

Experimental system. Figure 2A shows a simple kinetic scheme for the telomerase reaction with a primer with a TTGGGG 3′ end. The first three polymerizations onto such a primer are shown.

To analyze the kinetics of the telomerase reaction and sequence-dependent primer effects on the different steps shown in Fig. 2A, we compared kinetic parameters of sets of primers which differed only at the 5′ end, all having the same -TTGGGG 3′ end. Thus all primers had an identical degree of potential Watson-Crick base pairing with the telomerase RNA template domain (Fig. 1).

G-quartet formation by G-rich DNA oligonucleotides has been shown to inhibit their utilization as primers by telomerase from the ciliate *Oxytricha nova* (20). To avoid this complication, short oligonucleotides which cannot form intramolecular G-quartets were used in these experiments and the reaction buffer contained no added monovalent metal ions and a low (0.5 mM) Mg^{2+} concentration. Furthermore, immediately before it was added to the reaction, oligonucleotide was heat denatured and briefly preequilibrated at the reaction temperature for a time too short to allow significant intermolecular G-quartet formation (14, 20). The reaction times used (typically under 10 min) were also short enough to preclude such G-quartet formation.

Kinetic analyses were performed with octyl-Sepharose-purified telomerase with low residual nuclease activity and negligible other DNA polymerase activity (see Materials and Methods). Because of the availability of limited amounts of enzyme, initial reaction rates were measured under steady-state conditions. The 5-min reaction time for the reactions from which kinetic parameters were typically measured was confirmed to fall well within initial (linear) reaction rates (12a).

Kinetics of the T-only reaction. When dTTP is the only triphosphate substrate present (i.e., the T-only reaction), only two T residues (t1 and t2) can be added to a primer with a TTGGGG 3′ end (Fig. 2B). For the majority of primers used in this study, both t1 and t2 bands were prominent on a sequencing gel. This indicated that after t1 product formation, the enzyme either pauses or dissociates and reinitiates.

Direct estimation of the amount of active telomerase enzyme present in the reaction (see below) and quantitation of the radioactive signal in the t1 and t2 products after 2- to 3-min reactions showed that the molar amounts of both these products were typically >100 times the amount of telomerase present. Therefore, the observed amounts of t1 or t2 products must have resulted from multiple turnovers.

A pulse-dilution-chase experiment was performed to directly test whether stalled telomerase reaction intermediates also contributed to the t1 product (Fig. 3, Table 1). Halfway through a 6-min reaction primed by $(TG)_3T_2G_4$, the reaction mix was diluted 20-fold into a mixture containing unlabeled dTTP, reducing the ^{32}P specific activity 20-fold but maintaining the same dTTP concentration (1.25 μM) throughout the reaction. This latter point is important because the ratio of t2 to t1 product changes with dTTP concentration (see below). By dilution of the enzyme and primer 20-fold, the probability of any reinitiations that would lead to increases in t1 and t2 signals after the dilution-chase was greatly reduced, since the concentration of the primer during the chase (20 nM) was well below its measured apparent K_m (K_m^{app}) of 100 nM (see below) under these conditions. If the reaction contained a significant amount of stalled telomerase-t1 product complex, then during the dilution-chase t1 would be chased into t2 and

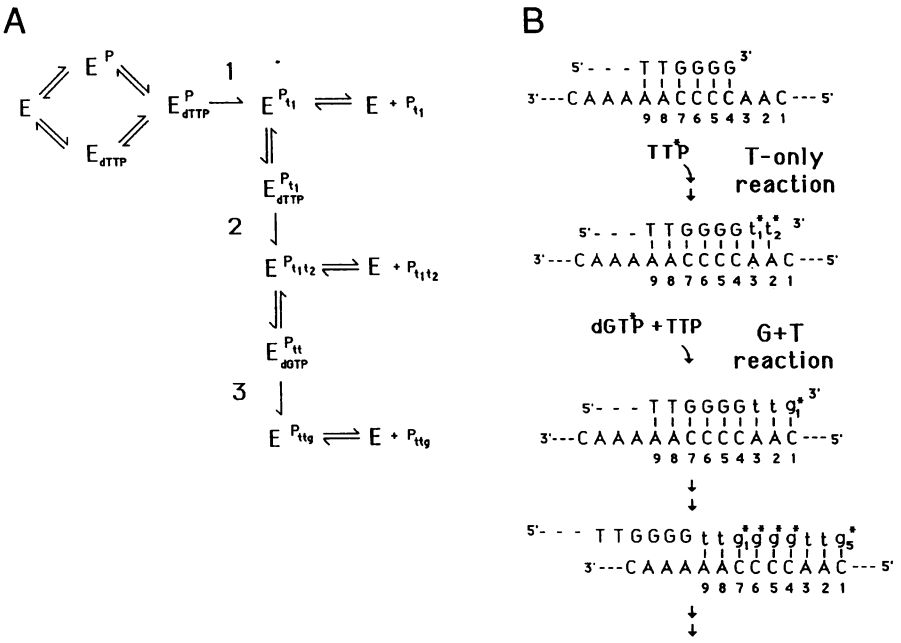

FIG. 2. (A) Simple kinetic scheme for telomerase reaction. The first three steps (marked 1, 2, and 3) are shown for a primer ending in -TTGGGG, 3′ end. After each polymerization, dissociation of product is shown as an alternate pathway to the right. E, telomerase; P, primer; P_t, P_{tt}, P_{ttg}, the products of the three polymerization steps. (B) Schematic of the reactions studied in this work. The template-forming sequence and addition products are numbered for reference in the text. The primer and RNA are in capital letters, and residues added are in lowercase letters. Lines represent base-pairing interactions. Asterisks denote ^{32}P label incorporation: in the T-only assay, from [α-^{32}P]dTTP with t1 and t2 products shown; in the G+T assay, from [α-^{32}P]dGTP. The first round of polymerization in the G+T reaction gives the g1 product, and after translocation to the 3′ end of the template, a subsequent cycle of polymerization gives the g5 product.

the t2/t1 ratio would increase. In the experiment whose results are shown in Fig. 3 and Table 1, no significant change in t2/t1 was observed (compare duplicate lanes 5 and 6 with lanes 1 and 2). Similar results were obtained with $G_4T_2G_4$ and $TGTGT_2G_4$ primers (data not shown).

We conclude that the vast majority of t1 product in the T-only reaction accumulates as the result of multiple turnovers of telomerase and primer rather than the stalling of paused telomerase-t1 product intermediates. In the kinetic scheme shown in Fig. 2A, there is a kinetic branch point after t1 formation (step 1); the t1 product can dissociate from telomerase or proceed to step 2. Because of the large molar excess of unreacted substrate primer over enzyme active sites in the T-only reaction (typically >1,000:1), effectively only the t1 products remaining associated with the enzyme elongate to form t2 product. The t2/t1 ratio then reflects the probability of proceeding to the next polymerization event, t2 formation (step 2 in Fig. 2A) on the original elongated primer, versus t1 product dissociation.

Effects of the primer 5′ end on T-only reaction kinetics. We determined the effect of varying the length of primers with the same TTGGGG 3′-end sequence on the kinetic parameters of the T-only reaction. V_{max} and K_m^{app} values were calculated from Eadie-Hofstee plots of the initial rates of t1 + t2 product formation (for calculations, see Materials and Methods) determined at several primer concentrations. As shown in Table 2, the addition of G_4 to the 5′ end of the 6-nt primer T_2G_4 resulted in a 5- to 10-fold decrease in the K_m^{app} and more than a 100-fold increase in the reaction V_{max}. Adding more T_2G_4 repeats to the 5′ end further decreased K_m^{app} as well as V_{max}, while the t2/t1 ratio increased. For short primers with relatively low t2/t1 ratios, the t2/t1 ratio decreased modestly with increasing primer concentration, whereas the opposite was seen for the 24-nt $(T_2G_4)_4$.

To determine the effect of the 5′-end sequence on the T-only reaction, we compared the set of four 12-nt primers listed in Table 3. For each primer, Fig. 4A shows an autoradiogram of the products separated on a DNA sequencing gel, Fig. 4B shows plots of the data, and Table 3 shows the calculated kinetic parameters. The primer $T_3A_3T_2G_4$ consistently had higher V_{max} and K_m^{app} values than did $G_4T_4G_4$, $(T_2G_4)_2$, and $(TG)_3T_2G_4$, whose K_m^{app} (0.1 to 0.2 μM) and V_{max} values were similar. The t2/t1 ratios also varied significantly (up to sixfold) among these primers (Table 3).

The results obtained with the seven 10-nt primers shown in Table 4 are presented in Fig. 5 and Table 5. Because of experimental variation in the activity of different telomerase preparations and between reaction cocktails, for Fig. 5 the primers were analyzed in sets of two or three with the same batch of complete reaction mix, and each pair of graphs shows a common primer to normalize the results obtained between experiments. Thus, accurate comparisons could be made among all the primers. The V_{max} values shown in Table 5 were obtained by analyzing all the primers shown together in the same experiment. For these 10-nt primers, with 1.25 μM dTTP the t2/t1 values were low (ranging from ~ 0.05 to 0.15) and comparisons were subject to error; at 10 μM dTTP the t2/t1 ratios were all proportionally increased (about threefold) and could be quantitated more accurately. Under these conditions the t2/t1 ratios showed up to fourfold differences among these primers (Table 5). At 10 μM dTTP, however, primer-dependent differences in the shapes of the saturation curves were less pronounced than those shown in

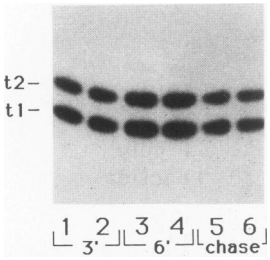

FIG. 3. t1 products dissociate rather than pause as intermediates. Lanes appear in duplicate pairs. The T-only reaction with 1.25 μM [α-^{32}P]dTTP (400 Ci/mmol) and 400 nM $(TG)_3T_2G_4$ primer was stopped after 3 min (lanes 1 and 2) and 6 min (lanes 3 and 4). In lanes 5 and 6, the reaction was diluted 20-fold with respect to enzyme, primer, and [α-^{32}P]dTTP specific activity after 3 min, and the reaction was stopped at 6 min. t1 and t2 product bands are marked to the left of the gel. Quantitation of results is presented in Table 1.

TABLE 1. Quantitation of results presented in Fig. 3[a]

Reaction time (min)	Concn (10^{-1} fmol) of[a]:		t1/t2[a,b]
	t1	t2	
3	5.3 (0.5)	3.9 (0.2)	0.75 (0.01)
6	9.9 (0.2)	7.4 (0.2)	0.73 (0.01)
3 (chase) + 6 (stop)	4.1 (0.9)	3.2 (0.5)	0.76 (0.03)

[a] Mean of duplicate lanes; standard deviations are in parentheses.
[b] t2/t1 was calculated for each lane and then averaged.

Fig. 5, which were obtained with 1.25 μM dTTP. The significance of these dTTP concentration effects is discussed below.

The effects of the 5'-end primer sequence on V_{max} and t2/t1 were more marked with the 10-nt primers than with the 12-nt primers (Fig. 5). For example, the V_{max} of CACA- was 10-fold greater than that of CAAA- (Fig. 5A), yet these primers differ by only one base. For most of the 10-nt primers tested, when they were assayed with 1.25 μM dTTP, the measured K_m^{app} values were similar, taking into account the calculated error, and ranged from 0.3 to 0.9 μM. However, the K_m^{app} of $CACAT_2G_4$ (2.4 μM) was again significantly different from those of the other 10-nt primers analyzed (Table 5).

These results show that interactions between the primer and telomerase that are distinct from simple base pairing of the 3' end with the telomerase RNA template significantly affect the kinetics of the T-only reaction.

With short primers, V_{max} is not determined by the rate of product dissociation. The observed V_{max} or k_{cat} of an enzymatic reaction is determined by the rate-limiting step in the reaction. To narrow the possibilities for the step(s) that accounts for the observed differences in V_{max} among the 10-nt primers, we used the procedure of Reardon and Miller

TABLE 2. Effects of primer length on T-only reaction

Primer sequence	K_m^{app} (μM)	Relative V_{max}	t2/t1[a]
T_2G_4	2.23 ± 0.31[b]	<0.01	ND[c]
$G_4T_2G_4$	0.39 ± 0.25	1	0.15–0.12
$(T_2G_4)_2$	0.06 ± 0.03	0.5	0.46–0.36
$(T_2G_4)_3$	0.02 ± 0.01	0.25	0.92–0.92
$(T_2G_4)_4$	0.03 ± 0.02	0.2	0.87–1.05

[a] t2/t1 ratios are given as the range of values calculated at each primer concentration: 7.5, 15, 31, and 62.5 nM for $(T_2G_4)_3$ and $(T_2G_4)_4$ and 62.5, 125, 250, and 500 nM for $G_4T_2G_4$ and $(T_2G_4)_2$.
[b] T_2G_4 was tested in a separate experiment alongside $G_4T_2G_4$. K_m^{app} for $G_4T_2G_4$ in both experiments was identical (within experimental error).
[c] ND, not determined.

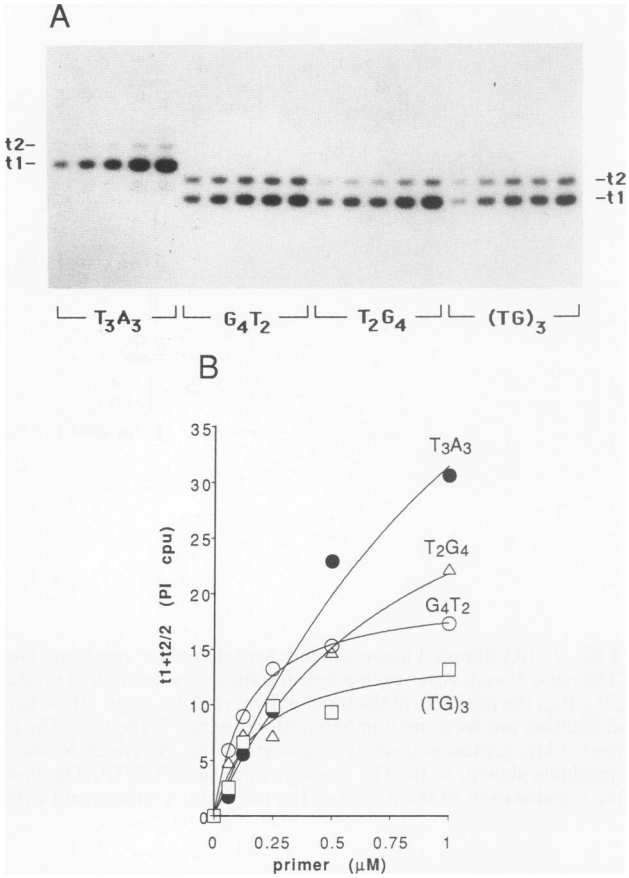

FIG. 4. Effect of the 5'-end sequence of 12-nt primers on the T-only reaction. (A) Telomerase reaction products under standard T-only assay conditions with 0.0625, 0.125, 0.25, 0.5, and 1.0 μM (shown from left to right on gel) each 12-nt primer. (B) Radioactivity incorporated (expressed as PhosphorImager counts [PI cpu]) from the two bands (t1 + 0.5t2; see Materials and Methods) was plotted against primer concentration. K_m^{app}, relative V_{max}, and t2/t1 ratios obtained from panel B and Eadie-Hofstee analysis of data are presented in Table 3.

TABLE 3. K_m^{app}, relative V_{max}, and t2/t1 values from Fig. 4B and Eadie-Hofstee analysis of data

Primer (5' end)[a]	K_m^{app} (μM)	V_{max} (relative)	t2/t1[b]
T T T A A A	ND	>3[c]	0.05–0.09
T T G G G G	0.16 ± 0.10	1.4	0.18–0.25
G G G G T T	0.22 ± 0.12	1.4	0.38–0.68
T G T G T G	0.15 ± 0.01	1	0.41–0.56

[a] All primers have a common -TTGGGG 3' end. Residues that can form base pairs with RNA near the template-forming region are underlined.
[b] The t2/t1 ratios were calculated for each primer concentration and are shown as the range of values from low to high primer concentration.
[c] V_{max} for T_3A_3 was estimated from the graph in Fig. 4B because the datum points in this and other experiments were not linear in an Eadie-Hofstee plot. In an earlier experiment the relative V_{max} of $T_3A_3T_2G_4$ was sixfold higher than those of $G_4T_2G_4$ and $(T_2G_4)_2$.

TABLE 4. Sequences of 10- and 12-nt primers analyzed[a]

RNA template sequence	-6 -5 -4 -3 -2 -1 9 8 7 6 5 4 3 2 1
	3'...T C T A A A A C C C C A A C...5'
10-nt primers	5' T T T T T G G G G 3'
	T G T G
	G G G G
	T A A A
	C A A A
	C A C A
	C C C A
12-nt primers	G G G G T T
	T G T G T G
	T T G G G G
	T T T A A A

[a] The common -TTGGGG 3' end is written out for the first primer only. These six bases can base pair with the template-forming sequence of the telomerase RNA, shown and numbered on the top line for reference. Other bases in the 5' end of the primer that can base pair with the RNA region 3' to the template sequence are underlined.

for human immunodeficiency virus reverse transcriptase (15) to test whether V_{max} is determined by rate-limiting product dissociation. In these experiments, if a rate-limiting step occurs after product formation, the plot of product formation versus time is biphasic, with an initial burst of product formation resulting from the first enzyme-primer turnover event; a rate-limiting step (such as product dissociation) that occurs after the chemical step (product formation) determines the later, lower steady-state rate. Under rapid-reaction-rate conditions, the initial burst phase is not resolved but is indicated by intersection above zero on the y axis of the time course plot of product formed during the steady-state phase of the reaction.

With the 24-nt primer $(T_2G_4)_4$, from 0.5 to 2 min, the rates of product formation were linear and proportional to the amount of enzyme present (Fig. 6A). In every experiment, for each enzyme concentration tested, extrapolation of this linear portion of each time course plot back to 0 min reproducibly intersected the y axis above zero (Fig. 6A). This experiment was repeated four times, with the reactions in one experiment being scaled up twofold to limit further the experimental scatter in the plots. We concluded that the primer $(T_2G_4)_4$ showed biphasic burst kinetics. These results provide strong evidence that, for this primer, product dissociation is rate limiting. The low measured K_m^{app} for $(T_2G_4)_4$ (Table 2) and the processive action of telomerase that has been reported previously with this primer (6) are both consistent with this conclusion.

Having established that the telomerase T-only reaction primed by $(T_2G_4)_4$ shows biphasic kinetics, we tested whether t1 product dissociation was rate limiting for $G_4T_2G_4$, $CACAT_2G_4$, $TGTGT_2G_4$, and T_6G_4 under the same reaction conditions. These primers were chosen for comparison since among the 10-nt primers they covered the widest range of measured reaction rates (k_{cat} or V_{max}, as measured in this work) and K_m^{app} values. Again, product formation was linear with time and proportional to the amount of enzyme present (Fig. 6B). With each of these 10-nt primers, the linear plots intersected close to or even slightly below zero. Intersection on the y axis below zero was unlikely to be caused by rate-limiting initial primer-enzyme complex formation. First, enzyme and primer were preincubated prior to starting the reactions. Second, y-intercepts below zero most often occurred with the highest enzyme concentration, when it would be least likely that complex formation was rate limiting. As shown in Fig. 6B, in no experiment with a 10-nt primer did any of the lines extrapolate significantly above zero on the y axis. These results were in sharp contrast to those with the $(T_2G_4)_4$ primer.

These findings showed that although a post-product formation step such as product dissociation is rate determining for the 24-nt primer $(T_2G_4)_4$, it is not rate determining for the four 10-nt primers tested under the same conditions. Hence, for the 10-nt primers, the observed differences in the rate of the T-only reaction under steady-state conditions are determined by a step(s) prior to or concomitant with product formation.

The initial stoichiometric burst of product formation observed with $(T_2G_4)_4$ as primer allowed us to calculate the active-site concentration of telomerase in the octyl-Sepharose fractions. The replot of y-axis intercepts (product formation from the first enzyme-primer turnover event) against volume of enzyme is shown in the inset in Fig. 6A. From the slope of this replot, the active-site concentration was calculated to be 6 ± 2 pM. This calculation gives a minimal number of active sites, since it assumes that every telomerase-primer complex formed yields product.

Analysis of t2/t1 ratio and V_{max} trends among the 10-nt primers further reinforced the interpretation that the differences in V_{max} of t1 formation among the 10-nt primers are not caused by differences in a rate-limiting product dissociation step. First, in the reaction scheme shown in Fig. 2A, there is a kinetic branch point following step 1. V_{max} for t1 formation and the t2/t1 ratio would be inversely related if the rate constants for steps 1 and 2 were constant between primers and if dissociation of t1 product were rate limiting, since a decreased probability of t1 dissociation would result in a proportionally higher probability of elongation of t1 to form t2. However, among the 10-nt primers the relative V_{max} and t2/t1 ratios did not vary inversely; $TGTGT_2G_4$ and $G_4T_2G_4$ had both high t2/t1 ratios and high V_{max} values (Table 5).

Second, the relative rates of product dissociation would be expected to reflect the relative affinities of telomerase for the different primers. One possible determinant of this could be the extent of potential additional base pairing beyond the TTGGGG 3' end. However, this clearly was a poor predictor of V_{max} or t2/t1 ratios. The G-rich primers $TGTGT_2G_4$ and $G_4T_2G_4$ were again the exceptions. Beyond the T_2G_4 3' end, $TTTTT_2G_4$ can make 3 extra base pairs of a continuous template RNA-DNA primer helix (underlined) (Fig. 2B), yet its t2/t1 ratio was the same as that of $TGTGT_2G_4$, which has only one additional potential base pair. Furthermore, none of the five other 10-nt primers tested has any potential for additional base pairing, yet $G_4T_2G_4$ had both a higher t2/t1 ratio and a higher V_{max} than $CAAAT_2G_4$ and $TAAAT_2G_4$.

From the absence of an initial burst in the reaction time courses for the four 10-nt primers tested, we conclude that the rate-limiting step in the T-only reaction occurs before product dissociation. The specific kinetic parameters that might be affected by the 5' end of the primer are considered in the Discussion. On the other hand, for the long primer $(T_2G_4)_4$ we found evidence that product dissociation is the rate-limiting step. These results were consistent with the differences in K_m^{app} values of these primers: 30 nM for $(T_2G_4)_4$ (Table 2) compared with >300 nM for the 10-nt primers.

Kinetics of synthesis of complete TTGGGG repeats. The

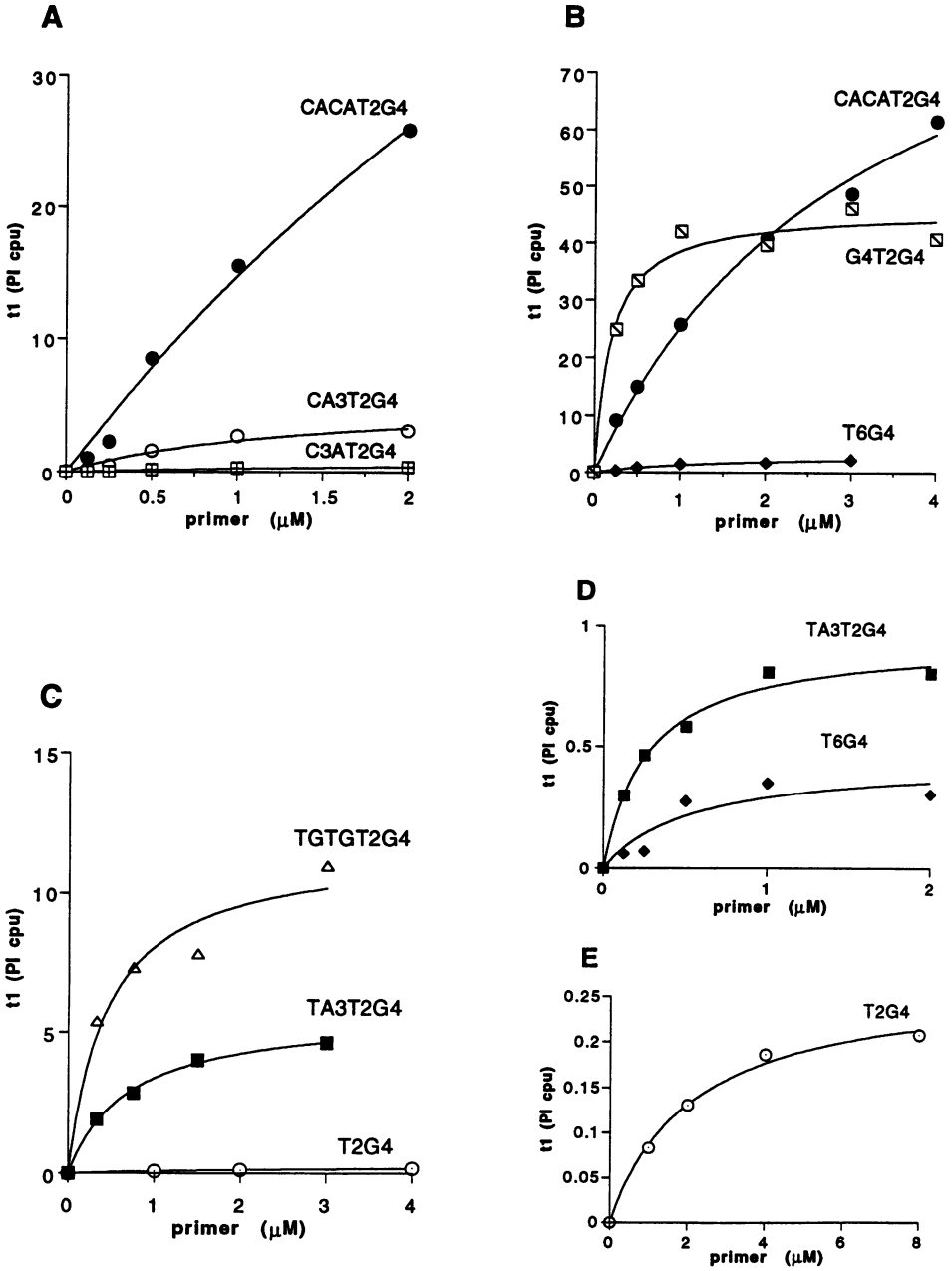

FIG. 5. Dependence of T-only reaction on 5′-end sequence of 10-nt primers. (A to D) Each panel represents a single experiment. Radioactivity incorporated in t1 bands was quantitated by PhosphorImager and plotted against increasing primer concentration in standard T-only reactions with 1.25 μM [α-^{32}P]dTTP. (E) The *y*-axis scale of the plot of T_2G_4 from the experiment in panel C is enlarged to show the primer saturation curve more clearly.

T-only reaction described above can be considered an "abortive initiation" reaction in which, after step 2 in the kinetic scheme in Fig. 2A, the absence of dGTP forces the reaction along the product dissociation pathway. In the presence of 1 to 2 μM [α-^{32}P]dGTP, saturating (100 μM) dTTP, and primer, a typical in vitro reaction yields products with complete T_2G_4 repeats that are up to hundreds of residues long after a 10- to 20-min incubation. We refer to this reaction as the G+T reaction.

Synthesis of the third addition product from a primer with a T_2G_4 3′ end involves copying the 5′-most C residue of the RNA template (template position C1 [Fig. 2B]) (9). This has recently been proven directly (12b). As after steps 1 and 2 in Fig. 2A, a kinetic branch point exists after the g1 product is formed: product can dissociate or can translocate to the 3′ portion of the template and be elongated. Hence, this "g1" product will be referred to as a translocation G product. Because of the large excess of primer in the reactions, only g1 product remaining associated with the telomerase enzyme will be detectably elongated. The next translocation G product, g5 (containing five ^{32}P-labeled G residues), results from the first round of synthesis after translocation of the g1

TABLE 5. Relative V_{max} and t2/t1 ratios for 10-nt primers with a T_2G_4 3′ end

Primer[a]	Relative V_{max}[b]	t2/t1[b,c]	K_m^{app}[d]
C C C A	ND[e]	ND	ND
T T T T	1	0.55	0.3 (0.5)
T A A A	2	0.25	0.45 (0.2)
C A A A	3.2	0.24	0.9 (0.3)
T G T G	3.3	0.59	0.4 (0.1)
G G G G	5.6	0.35	0.3 (0.1)
C A C A	6.4	0.15	2.4 (0.3)

[a] 5′ end shown; underlined residues can pair with RNA bases in the template-forming region.
[b] Both t2/t1 and relative V_{max} values were determined from experiments with 10 μM dTTP.
[c] t2/t1 ratios are averages of duplicates at 1 μM primer concentration. The error (standard deviation) range was 0.001 to 0.02.
[d] K_m^{app} was determined from experiments with 1.25 μM dTTP. Standard deviations are given in parentheses.
[e] ND, not determined because of very low incorporation under the assay conditions used.

product. Longer products can form by subsequent cycles of translocation-elongation (Fig. 2B).

Nonprocessivity of telomerase with short primers. In the T-only experiments described above, we found a high probability of t1 product dissociation. Such low processivity could have been attributable to the subsaturating dTTP concentrations used in these experiments (the K_m^{app} for dTTP is ~5 μM). This was consistent with the finding that t2/t1 increased with dTTP concentration. Therefore we analyzed t1 product dissociation in the presence of saturating dTTP. To obtain sufficient label in t1 and t2 products, the G+T reaction was performed with a primer ending in T_2G_3. As shown previously (8), telomerase extends such a primer by addition of an initial ^{32}P-labeled dG residue (*g product). Hence, the next two products, *gt and *gtt, formed by additions of T residues, now contain radiolabel and are detectable. Figure 7A shows a time course of the G+T assay on the 11-nt primer $T_2G_4T_2G_3$. The overall incorporation of radioactive label was linear for 20 min. Not only did the average length of products increase with time, but also short products up to 21 nt in length increased in abundance over time. In the extreme case, if the telomerase reaction were completely processive, no multiple turnovers on primer would occur during the course of the reaction. In this situation, band intensity would increase directly with product length, since each additional incorporation of [α-^{32}P]dGTP onto the 3′ end of the growing product adds one more radioactive ^{32}P signal onto the previous product. However, in Fig. 7A it is clear that at every time point the shorter products, especially those up to 15 nt long, are more abundant than the successively longer products on the gel. As in the T-only reactions, the molar amounts of these short products typically were at least 2 orders of magnitude greater than the amount of telomerase present. Therefore, accumulation of paused telomerase-short product intermediates cannot account for the great majority of the signal seen.

Pulse-chase experiments confirmed that the intensity of short product signals is due to multiple rounds of telomerase dissociation and reinitiation rather than to stalled telomerase-product complexes. The 10-nt $G_4T_2G_4$ was used as the primer. After labeling with [α-^{32}P]dGTP, a G+T reaction was chased with unlabeled dGTP (Fig. 7B and C). Again, without a chase, all products shorter than 20 nt accumulated significantly between 2.5 and 13 min (compare duplicate lanes 1 and 2 with 3 and 4 in Fig. 7B, and gel scans of lanes 1 and 3 in Fig. 7C). Note that the scale of the lane 1 scan is expanded twofold relative to those of the lane 3 and 5 scans). These <20-nt bands also increased in intensity even when the reaction was chased with a 10-fold excess of unlabeled dGTP (compare lanes 1 and 2 with lanes 5 and 6 and corresponding scans). These experiments showed that the short products could not be chased into longer products, as would be expected for paused intermediates.

Altogether, these results indicated that the short products in the G+T reaction result from significant numbers of enzyme turnovers during the reaction, reflecting a high probability of product dissociation rather than proceeding to the next polymerization event on the original primer (see the kinetic scheme in Fig. 2). This result is especially striking for the *g and *gt products in Fig. 7A, each of which precedes addition of a T residue, because dTTP was saturating.

Variable amounts of labeled 11- and 12-nt products were detected in G+T reactions with the 10-nt primers. However, as described above, the only labeled triphosphate in the reaction was [α-^{32}P]dGTP, and the shortest product containing label should have been the 13-nt tt*g. We have shown that the nuclease levels in the telomerase preparations are too low for primer degradation to account for these products (Materials and Methods). 5′-3′ exo- or endonuclease activity would predict label in products of 10 nt or less, which was not observed. We propose that these 11- and 12-nt labeled products arise from mispositioning of the 3′ end of the primer on the template. Since they represent abortive products and do not contribute to the majority of the elongation products, their production does not interfere with the conclusions made here.

In contrast to the results with shorter products, the label in ~30- to 50-nt products could be chased with cold dGTP into longer products (Fig. 7B and C, long-product regions of the lane 5 scan). These results indicated that these ~30- to 50-nt products have a relatively high probability of remaining associated with telomerase; hence, the reaction becomes more processive with increasing product length. However, above ~23 nt, labeled individual elongation products, corresponding to copying of different template positions, were differentially depleted over time or with a cold dGTP chase. Compared with neighboring bands, the label in the 31-, 37-, and 43-nt translocation G products was not depleted over time and much less label was chased into longer products. In contrast, the bands in the 30- to 50-nt size range corresponding to addition of the three G residues after translocation (from copying positions C-6, C-5, and C-4) were depleted with time and also chased into longer products. The 24-, 30-, and 36-nt products correspond to copying template position A2 (Fig. 2B). The label in these bands was not depleted over time without a chase (Fig. 7B, lanes 3 and 4) but was depleted with the cold-dGTP chase (lanes 5 and 6). The K_m^{app} for dGTP is estimated to be ~1 μM (see Materials and Methods). These results imply that at the initial dGTP concentration (1.25 μM), addition of the next residue, the translocation G, from copying position C-1, is rate limiting and that these telomerase-product complexes pause until dGTP binds.

In summary, under these reaction conditions the processivity of the telomerase reaction increased with product length once the elongation products reached the 30- to 50-nt range. The specific persistence of the translocation G bands after a chase suggested that in this size range the highest probability of product dissociation occurs after copying the C-1 residue.

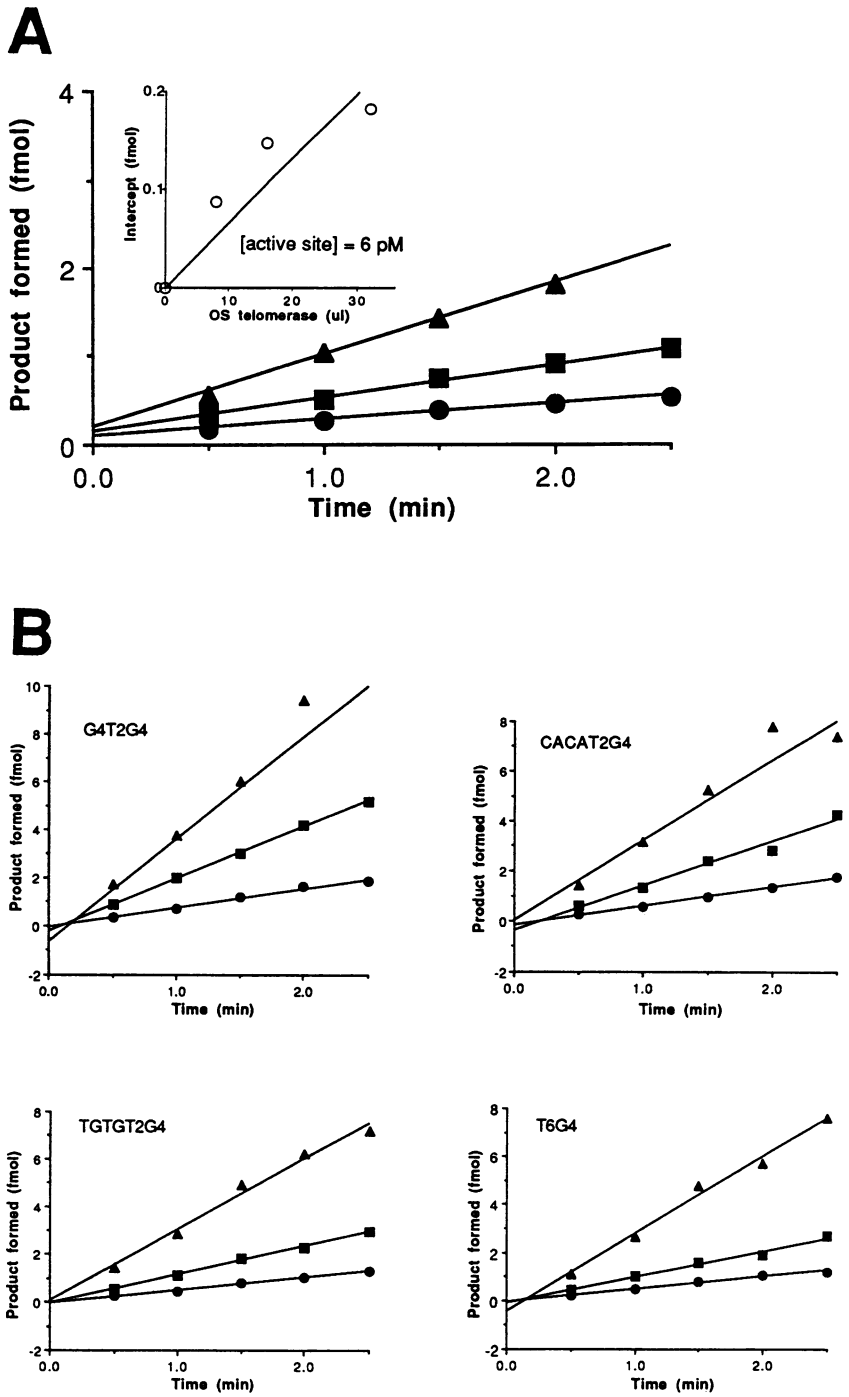

FIG. 6. Time course of dTTP incorporation to test for biphasic kinetics of telomerase reaction. We mixed 8 (●), 16 (■), or 32 (▲) μl of telomerase preparation with 8 μM primer and 10 μM [α-^{32}P]dTTP. Aliquots from the total reaction mixtures were stopped after 0.5, 1.0, 1.5, 2.0, and 2.5 min. (A) Time course plot with primer $(T_2G_4)_4$. Inset: *y*-intercepts from the equations to the lines drawn in panel A were converted from PhosphorImager units to moles of product formed and plotted against the amount (in microliters) of telomerase used per reaction. The slope of the line gives a minimal estimate (see text) of the telomerase active-site concentration. (B) Time course plot of dTTP incorporation performed as in panel A for the primers $G_4T_2G_4$, $CACAT_2G_4$, $TGTGT_2G_4$, and T_6G_4.

Effects of the primer 5′ end on kinetics of TTGGGG repeat synthesis. We determined whether the 5′ end of the primer with a T_2G_4 3′ end continues to affect the kinetic parameters as the elongation reaction proceeds further along the template and in subsequent posttranslocation steps. The same sets of oligonucleotides (Table 4) that had been compared in the T-only reaction were assayed in the G+T reaction. The profiles of products formed over a range of primer concentrations are shown for four of the 10-nt primers in Fig. 8A and for the 12-nt primers in 8C. Figure 8B compares all

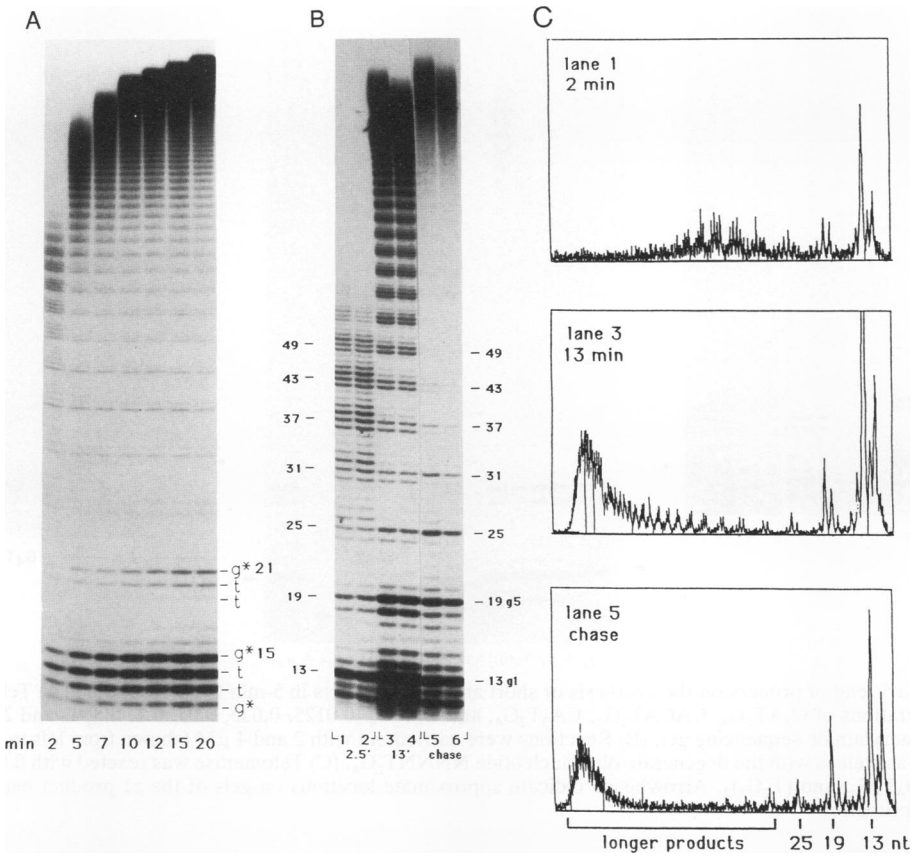

FIG. 7. G+T reaction time course and pulse-chase experiments. (A) Time course of G+T reaction with $T_2G_4T_2G_3$ as the primer. Telomerase was reacted with 30 nM $T_2G_4T_2G_3$, 1.25 μM [α-^{32}P]dGTP (400 Ci/mmol), and 100 μM dTTP in a 120-μl reaction mixture. Aliquots were removed into stop mix at 2-, 5-, 7-, 10-, 12-, 15-, and 20-min time points. Added residues and product lengths are marked. (B) Pulse-chase of G+T reaction. Lanes are in duplicate pairs. G+T telomerase reactions with 1 μM $G_4T_2G_4$ primer were allowed to proceed for 2.5 (lanes 1 and 2) and 13 (lanes 3 and 4) min. In lanes 5 and 6, G+T reactions were chased with cold dGTP added to a final concentration of 7.5 μM at 2.5 min. Reactions were chased for an additional 11.5 min. Numbers corresponding to the product sizes appear to the right of the gels. (C) PhosphorImager scans were generated from the gel in panel B. The *y*-axis scale of the scan of lane 1 is approximately double that of the lane 3 and 5 scans.

seven 10-nt primers, as well as a degenerate primer $N_4T_2G_4$, at 2 and 4 μM. Among these primers, the G+T reaction products differed in several ways.

First, we analyzed the rate of formation of the first translocation G product, g1, for each primer. This product can be analyzed directly because, as described above, under these reaction conditions the vast majority of product formed represents multiple enzyme turnovers and only a minor fraction is depleted into longer products. Specifically, the radioactivity incorporated into g1, g5, and longer products was quantitated by PhosphorImager analysis and corrected for the length of the products to determine the relative number of moles of product molecules formed. For the 10- or 12-nt primers, the g1 products arising from multiple turnovers represented >90% of the total reaction product molecules. Hence, for these primers the rate of formation of the g1 product is minimally affected by depletion into longer products.

Figure 9 shows the amount of g1 product formed plotted as a function of primer concentration for the 10- and 12-nt primers, using the data in Fig. 8A and C. As can also be seen from the shapes of these curves, primers again differed in both V_{max} and K_m^{app} values (this was confirmed by calculations from Eadie-Hofstee plots [data not shown]). If the 5′ end of the primer affected all the polymerization steps along the template equally, the differences in V_{max} for g1 formation might have been expected to reflect the differences in kinetic parameters seen in the T-only reaction: for example, a high V_{max} for t1 and t2 formation, and/or a high t2/t1 ratio, might predict a correspondingly high V_{max} for g1 formation. With the 12-nt primers, the V_{max} values for g1 formation inversely followed the trend of t2/t1 ratios in the T-only reaction, with $T_3A_3T_2G_4$ and $G_4T_4G_4$ having the lowest and highest V_{max} of g1 formation, respectively. However, among the 10-nt primers there were clear departures from this prediction. The V_{max} of g1 formation for $CA_3T_2G_4$ was higher than that for $G_4T_2G_4$ (Fig. 9A), whereas in the T-only assay both the V_{max} of t1 formation and the t2/t1 ratio for $CA_3T_2G_4$ were significantly lower than those for $G_4T_2G_4$ (Table 5). Thus, the effect of the 5′ end of the primer after step 2 in the kinetic scheme in Fig. 2 did not necessarily parallel the effect on the kinetics of t1 and t2 product formation. This difference could be exerted at either step 3 or the dissociation of t2, or both (Fig. 2A).

The degenerate primer $N_4T_2G_4$ was tested to analyze competition between the 4^4 different possible primer se-

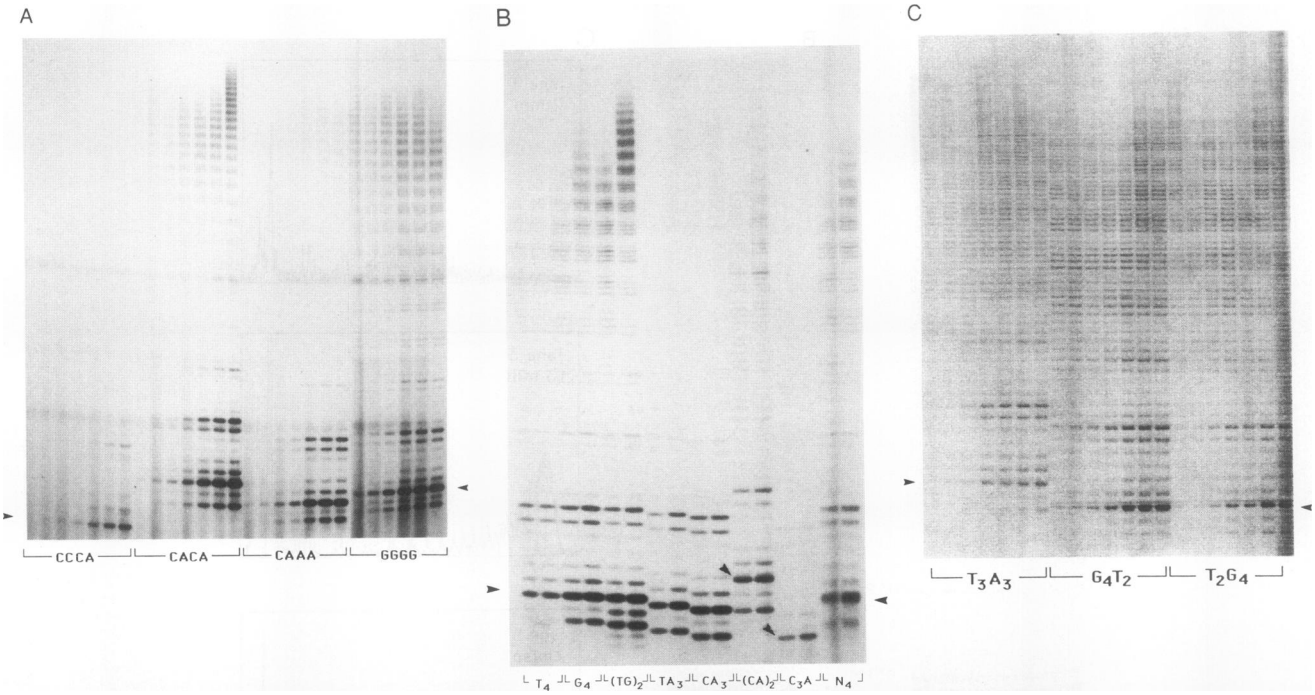

FIG. 8. Effects of the 5′ end of primers on the synthesis of short and long products in 5-min G+T reactions. (A) Telomerase was reacted with increasing concentrations of $C_3AT_2G_4$, $CACAT_2G_4$, $CA_3T_2G_4$, and $G_4T_2G_4$ (0.0125, 0.025, 0.05, 0.1, 0.5, 1, and 2 μM). Products were resolved on a 12% polyacrylamide sequencing gel. (B) Reactions were carried out with 2 and 4 μM (shown from left to right on the gel) each 10-nt primer in Table 2, as well as with the degenerate oligonucleotide $NNNNT_2G_4$. (C) Telomerase was reacted with 0.0625, 0.125, 0.25, 0.5, and 1 μM $T_3A_3T_2G_4$, $G_4T_4G_4$, and $(T_2G_4)_2$. Arrowheads indicate approximate locations on gels of the g1 product band, whose mobility is different with different primers.

quences present in the synthesized mixture. The amount of product formed from any one primer in the mixture is a measure of its k_{cat}/K_m compared with this parameter for all the other primers (5). The mobility of the g1 product of the other primers varied widely depending on the sequence of the input primer (Fig. 8B, arrowheads). In contrast, a very limited subset of mobilities was seen for the g1 product of the $N_4T_2G_4$ primer mixture. In particular, the $N_4T_2G_4$ g1 product band comigrated with those of T- and G-rich primers $T_4T_2G_4$, $G_4T_2G_4$, and $TGTGT_2G_4$ (Fig. 8B). This argues that there is strong discrimination among 10-nt primers with a common T_2G_4 3′ end.

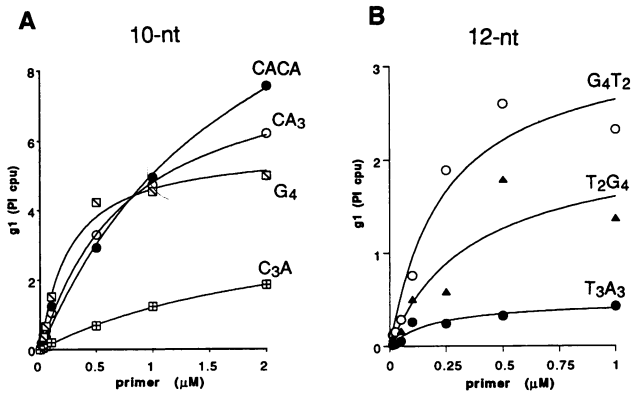

FIG. 9. Quantitation of g1 bands from the gels in Fig. 8, plotted against primer concentration for 10-nt (A) and 12-nt (B) primers. PI cpu, PhosphorImager counts.

We also analyzed the amounts of "long" products synthesized from the various primers. The ratio of longer products to g1 products is significant because it provides a measure of the "efficiency" of a primer in forming complete TTGGGG repeats. Although the long products result from several distinct polymerization and translocation steps, a key determinant of the long/g1 product ratio is likely to be the relative rates of the two pathways following the kinetic branch point after step 3 in Fig. 2: translocation-polymerization versus g1 product dissociation. As discussed in relation to Fig. 7, products in the ~25- to 50-nt range represent a zone of accumulation from shorter products and depletion into longer products. Therefore, to simplify analysis we considered products greater than 50 nt to be long products; thus we are measuring the proportion of reactions that become effectively fully processive.

As in the T-only reaction, the amounts of long product formed showed greater differences between the 10-nt primers than between the 12-nt primers (Fig. 8). By measuring the amounts of long products formed under V_{max} conditions, the 10-nt primers were ranked in the order C_3A- < TA_3- ~ CA_3- < T_4- < CACA- ~ G_4- ~ TGTG-T_2G_4. This ranking did not simply parallel the relative V_{max} values for g1 formation. Although T_4- yielded relatively small amounts of both long and g1 products compared with TGTG- and G_4-, at all primer concentrations tested T_4-, TGTG-, and G_4- had higher long/g1 product ratios than CA_3- and TA_3- did (Fig. 8B). Among the 12-nt primers, $T_3A_3T_2G_4$ had the lowest V_{max} of g1 product formation but a high long/g1 product ratio.

Furthermore, the long/g1 product ratio did not always reflect the t2/t1 ratio in the T-only assay: $T_3A_3T_2G_4$ had the lowest t2/t1 ratio of the 12-nt primers but, as mentioned

directly above, a high long/g1 product ratio at all primer concentrations. Likewise, $CACAT_2G_4$ had the lowest t2/t1 ratio of all the 10-nt primers tested, and yet in the G+T assay the long/g1 product ratio was high and comparable to that for $G_4T_2G_4$ (Fig. 8A).

DISCUSSION

By kinetic analyses we have shown that the 5′ end of the DNA primer affects the elongation reaction carried out by the *T. thermophila* telomerase. The primer can affect the reaction kinetics in the following different ways: a difference in the affinity of telomerase for the different primers, and/or rate differences in one or more steps in the reaction pathway. We found major differences among primers in V_{max} values and t2/t1 ratios, which, being measured at saturating concentrations of primer, are not affected by initial primer binding. For the 10-nt primers, we showed that product dissociation was not rate limiting. We conclude that the 5′ end of the primer may affect the reaction rate (k_{cat}) in a way that is not simply a consequence of different affinity for the enzyme. These findings provide the first evidence that the contacts made by the primer with the telomerase enzyme, in addition to the hybridization with the RNA template, impact on the polymerization by directly altering the reaction rate.

The primers used in this study had the same $-T_2G_4$ 3′ end, which can form up to a 6-bp DNA-RNA hybrid with the template sequence of the telomerase RNA. This ensured that the first sequence added to each primer was the same, making direct comparisons between primers possible. In the absence of such fixed template complementarity, we would anticipate that the kinetic differences between primers would be even greater than those found in the present study.

In vivo, the first telomeric sequence is often added onto nontelomeric ends completely lacking template complementarity, as in normal *Tetrahymena* development or unprogrammed chromosome healing events. However, in vivo studies suggest that telomere synthesis is distributive (18). Therefore, the results presented in this paper are directly informative in regard to telomere synthesis after the first one or a few repeats are added. The early stages of telomere synthesis may be critical, since it is expected that various nuclear activities can use a free DNA end as a substrate. For example, a DNA terminus in the nucleus is likely to be competed for by the type of telomere-binding protein found in some ciliates (14), other single-stranded DNA-binding proteins, nucleases of various kinds, and components of the recombination and possibly replication machineries, as well as by telomerase. Our results suggest that even after addition of one or a few repeats, the sequence 5′ to the end strongly influences whether the outcome is stable telomere addition. Specifically, the 5′-end sequence of the primer determined the probability of addition of the next nucleotide versus product dissociation in the T-only and G+T assays described in this work. In the G+T assays, the 5′ end ultimately determined the fraction of the reaction engaged in the synthesis of long products. Thus, even when the 5′ end of the primer is far beyond the active polymerization site, the primer continues to affect the level of synthesis of long products.

For all primers, with the addition of more repeats polymerization became more processive. This can be explained by increased binding of the 5′ portion of the growing primer to telomerase, possibly by base-specific contacts, as discussed below, causing a decreased probability of dissociation after each polymerization step. Directly supporting this interpretation, product dissociation was found to be rate limiting for the primer $(T_2G_4)_4$ but not for the 10-nt primers analyzed. Eventually, during telomere addition in vivo, once the healing end acquires many repeats, it will become indistinguishable from a natural end with respect to telomerase kinetics.

The results reported here can reconcile the apparent differences in the processivity of *Tetrahymena* telomerase observed in vivo and in vitro (6, 18). From in vitro studies by Greider (6), the processivity of telomerase was determined to be 521 nt, the number of nucleotides synthesized while half the bound telomerase molecules remain associated. Those studies were carried out with the primer $(T_2G_4)_4$. From our results we would expect the processivity of telomerase measured under those conditions to be less with shorter primers. In the in vivo studies (18), telomeres were cloned and sequenced from *Tetrahymena* cells expressing both mutant and wild-type telomerase RNAs. Mutant repeats were found highly interspersed among wild-type repeats, with about one repeat being synthesized on average before product dissociation. The interspersion data also showed that in vivo, telomerase continues to act in a nonprocessive manner even after addition of at least 10 repeats (18). The results reported in the present study show that for 10- to 12-nt DNA primers, during the initial steady-state conditions, even at saturating dNTP concentrations the vast majority of enzyme molecules are engaged in multiple turnover reactions producing short products. Together, these results suggest that, although telomerase is clearly capable of processive synthesis in vitro (6), in vivo synthesis may be predominantly distributive. The great majority of the protruding single-stranded T_2G_4 repeat ends in *Tetrahymena* cells are only 12 nt long (11). Therefore the failure to synthesize repeats processively in vivo may be attributable to rapid synthesis of the complementary C_4A_2 repeat strand, so that the substrate for telomerase is usually a relatively short protruding end. Additional factors such as competition by telomere-binding protein may contribute to the lack of processivity in vivo.

Our findings highlight the need to take into account the contribution of rate-limiting product dissociation when considering the efficiency of a primer. For example, in Table 2, the 6-nt T_2G_4 had the lowest V_{max}, the 10-nt primer $G_4T_2G_4$ had the highest V_{max}, and then V_{max} decreased with increasing length for the longer primers $(T_2G_4)_2$, $(T_2G_4)_3$, and $(T_2G_4)_4$. Since product dissociation is not rate limiting for the 10-nt primers, it is unlikely to be rate limiting for the shorter 6-nt T_2G_4 primer. Hence we propose that this primer has a low V_{max} because it lacks the 5′ nucleotides which promote enzyme activity. We propose that above 12 nt, the 5′ end of the primer interacts with telomerase such that its affinity increases with increasing primer length. Thus at some point in this series of primers the rate of product dissociation now becomes rate limiting. Superimposed on this effect of rate-limiting product dissociation must be the sequence-specific effects of the 5′ end of the primer, which, as we have shown for the 10-nt primers, have dramatic effects on the catalytic rate constant in the absence of rate-limiting product dissociation. The minimal length required for the 5′ interaction to make product dissociation rate limiting is likely to vary with the 5′-end sequence of the primer. Therefore, for the longer primers that have been compared in previous reports (3, 7–10, 13, 16, 17, 20), it is unclear which kinetic parameters were affected by the differences in primer sequence.

By using the telomerase from *Oxytricha* cells, which synthesizes T_4G_4 repeats, the opposite effect of increasing

the primer length has been reported for T_4G_4 and $(T_4G_4)_2$ primers (20), the equivalent of the one- and two-repeat T_2G_4 and $(T_2G_4)_2$ primers compared in the present work. The overall rate of addition of the first four T residues of the reaction was higher with T_4G_4 than with the $(T_4G_4)_2$ primer. This could be due to species-specific differences between the telomerases of *Tetrahymena* and *Oxytricha* cells. In addition, as discussed above, product dissociation could be rate limiting for the 16-nt but not the 8-nt primer, masking any possible inductive effects of the primer on the reaction rate.

The kinetic data so far do not allow us to determine which of the steps preceding product dissociation are affected by the interaction with the 5′ end of the shorter (10-nt) primers. By making base-specific contacts with either RNA or protein or both moieties of telomerase, the 5′ end could increase the rate of any one or more of these steps (Fig. 2A): some step required to make an active enzyme-substrate(s) complex, including possibly a conformational change of the enzyme-substrate(s) complex, the triphosphate-binding step, and chemical steps 1 and 2 themselves.

The observed effect of dTTP concentration on the differences between short primers offers support for the proposal that a step preceding the chemical step is affected by the primer 5′ end: the lower the dTTP concentration, the greater were the kinetic differences between primers. By lowering the dTTP concentration, the chemical step is slowed. If, after the initial primer-binding step there is a second primer-binding step that precedes the chemical step, slowing the chemical step will allow more time for the bound forms of the primer to equilibrate and dissociate before the chemical step, resulting in greater discrimination between primers at this step (12). One candidate for such a prechemical step affected by the 5′ end of the primer is a conformational change of the enzyme-primer complex. After initial binding of the primer, a conformational change may align the primer 3′ end and its base-paired RNA template properly into the enzyme active site. At each polymerization step, as the 3′ end of the primer is extended, the active site has to move along the template. Therefore, at each position on the internal RNA template of telomerase the primer 3′ end is in a unique geometry with respect to the rest of the enzyme. This may explain why, for example, the 5′ end of the primer had different effects on the rates of g1 and t1 formation. An alternative explanation for the observed effects of dTTP concentration on K_m^{app} and V_{max} differences is random bireactant kinetics. In such cases, the binding of one substrate affects the binding of the other and can result in the kinetic differences described. Further kinetic experiments can be done to distinguish between these interpretations.

At some point, for translocation and processive synthesis to occur, unwinding of the primer from the template-forming region is required. Mechanistically, one way in which interactions of the 5′ end of the primer increase k_{cat} is that the 5′ end of certain primers could promote unwinding of the primer DNA-template RNA helix in the region away from the primer 3′ end and that this unwinding could promote progress to the next nucleotide. Our results point to the existence of sequence elements (such as G richness) that promote product formation and consequently turnover and overall reaction rate. This idea is supported by the simultaneously high V_{max} and t2/t1 ratios for the 10-nt primers $G_4T_2G_4$ and $TGTGT_2G_4$ compared with those of all the other 10- and 12-nt primers (which in some instances have either a high t2/t1 ratio or a high V_{max} but never both). A telomerase ribonucleoprotein-primer G residue interaction that promotes unwinding or other conformational change is attractive in light of the naturally occurring G-rich composition of the telomere and the mechanistically important need for unwinding and dissociation from the template-forming region.

In agreement with these results, Harrington and Greider showed that 24-nt pBR sequence oligonucleotides could prime *Tetrahymena* telomerase once G-rich or telomeric sequences were substituted either at the 3′ or 5′ end (10). Previous results with this telomerase (3, 8), as well as results with the human telomerase (13), have led to a model involving two modes of DNA recognition: hybridization to putative template sequence and G richness. The present work provides the first information about the mechanistic basis for such recognition.

In summary, our results show that even for primers with the same extensive (6-bp) degree of template hybridization, the sequence of the 5′ end of the primer has a marked effect on the reaction kinetics. Thus, residues in the primer separated by 6 nt or more from the reactive 3′ end of the primer can exert striking effects on primer utilization. We have shown that the rate of a given polymerization step by telomerase under V_{max} conditions can vary by over an order of magnitude depending on the primer 5′ sequence. In vivo, such reaction rate differences are likely to be one decisive factor in determining the fate of a DNA terminus, telomeric or nontelomeric, for which more than one kind of activity can compete. When combined with the contribution of sequence-dependent affinity of a primer for telomerase, which is likely to be important given the effective low concentration of a telomere or DNA terminus in vivo, it is clear that sequence-dependent effects on the kinetic parameters of the telomerase reaction can potentially be a strong influence on telomere and DNA end recognition in chromosome maintenance and healing.

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