Requirements for the dGTP-dependent Repeat Addition Processivity of Recombinant *Tetrahymena* Telomerase*

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Telomerase is a reverse transcriptase responsible for adding simple sequence repeats to chromosome 3'-ends. The template for telomeric repeat synthesis is carried within the RNA component of the telomerase ribonucleoprotein complex. Telomerases can copy their internal templates with repeat addition processivity, reusing the same template multiple times in the extension of a single primer. For some telomerases, optimal repeat addition processivity requires high micromolar dGTP concentrations, a much higher dGTP concentration than required for processive nucleotide addition within a repeat. We have investigated the requirements for dGTP-dependent repeat addition processivity using recombinant *Tetrahymena* telomerase. By altering the template sequence, we show that repeat addition processivity retains the same dGTP-dependence even if dGTP is not the first nucleotide incorporated in the second repeat. Furthermore, no dNTP other than dGTP can stimulate repeat addition processivity, even if it is the first nucleotide incorporated in the second repeat. Using structural variants of dGTP, we demonstrate that the stimulation of repeat addition processivity is specific for dGTP base and sugar constituents but requires only a single phosphate group. However, all nucleotides that stimulate repeat addition processivity also inhibit or compete with dGTP incorporation into product DNA. By assaying telomerase complexes reconstituted with a variety of altered templates, we find that repeat addition processivity has an unanticipated template or product sequence specificity. Finally, we show that a novel, nascent product DNA binding site establishes dGTP-dependent repeat addition processivity.

The ends of chromosomes in most eukaryotes are capped with tandem simple sequence repeats. These telomeric repeats and their associated proteins are necessary and sufficient to distinguish a stable linear chromosome end from a highly unstable DNA break (reviewed in Ref. 1). However, telomeres are incompletely replicated by DNA-dependent DNA polymerases. The resulting loss of telomeric repeats with cell proliferation induces senescence or apoptosis of cultured human primary cells (reviewed in Ref. 2). Telomeric repeats eroded by proliferation can be restored by the enzyme telomerase, a specialized reverse transcriptase (RT)\(^1\) that uses a defined region within its integral RNA component to template telomeric repeat synthesis (reviewed in Refs. 3, 4). Although telomerases in most organisms recognize only established telomeres as substrates, ciliate telomerases also recognize nontelomeric sites of developmentally programmed chromosome fragmentation. This ciliate telomerase chromosome healing activity is required to generate a transcriptionally competent macronucleus containing thousands of amplified, telomere-capped minichromosomes (reviewed in Ref. 5).

Most biochemical characterization of telomerase has been done in ciliate systems because of the relative abundance of enzyme (reviewed in Ref. 6). Ciliate telomerases have been shown to catalyze at least three activities. In a standard reaction, the template is copied by successive dNTP additions to synthesize a telomeric repeat. Second, if dITTP is reduced or omitted, telomerase reiteratively copies a template cytidine residue to synthesize product DNA composed of poly(dG). This template slippage-dependent polynucleotide synthesis resembles that catalyzed by human immunodeficiency virus RT in the presence of MnCl\(_2\) and the absence of a complete set of dNTPs (7). Finally, telomerase can catalyze nuleolytic cleavage of substrates or products. All of these nucleotide addition and removal activities appear to occur at the same active site.

Endogenous *Tetrahymena thermophila* telomerase assayed in cell extracts can add hundreds of repeats to a single primer before product dissociation (8). This high degree of repeat addition processivity requires primer and product interaction with a template-independent substrate anchor site (9, 10). Under standard reaction conditions, a primer bound only by interaction at the template, such as T\(_2\)G\(_4\), will be elongated to the template 5'-end but then will dissociate from the enzyme when dissociated from the template. In contrast, longer primers such as G\(_5\)T\(_4\)G\(_4\) or (G\(_4\)T\(_2\))\(_3\) can remain bound at the anchor site even when dissociated from the template. With anchor site interaction, a product 3'-end released from the template 5'-end can reposition at the template 3'-end to allow processive repeat addition. Anchor site interaction also substantially decreases the K\(_m\) for primer in vitro (9, 10).

Recombinant *Tetrahymena* telomerase, composed of only the telomerase RNA and telomerase reverse transcriptase (TERT) subunits that are essential for activity in vitro, demonstrates a more limited repeat addition processivity than the endogenous enzyme (11). In addition, unlike the endogenous enzyme, recombinant telomerase repeat addition processivity requires high micromolar dGTP concentrations, much higher than the submicromolar dGTP concentration required for processive nucleotide addition within a repeat. In this dGTP-dependence of

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\(^1\) The abbreviations used are: RT, reverse transcriptase; TERT, telomerase reverse transcriptase; dITTP, deoxyinosine triphosphate; NPS, nascent product binding site; RNP, ribonucleoprotein; nt, nucleotides.
repeat addition processivity, recombinant Tetrahymena telomerase resembles the endogenous Euploides aediculatus and Chinese hamster ovary cell telomerases (12, 13). The extent of repeat addition processivity varies among telomerase enzymes of different species and also between telomerase complexes of the same species (14, 15). It seems most likely that dGTP-stimulated repeat addition processivity is an inherent property of all telomerase RNPs, with the species-specific addition of other substrate anchor sites in a telomerase holoenzyme conferring the differences in repeat addition processivity observed.

For all telomerases known to demonstrate repeat addition processivity, dGTP is the first nucleotide added to a product repositioned from the template 5′- to 3′-end. One possible model to explain the dGTP dependence of repeat addition processivity would be that the stimulatory dGTP binds at the active site and enhances the probability of its addition as the first nucleotide in the second repeat. Alternately, dGTP could interact with a site entirely separable from the active site to stimulate repeat addition processivity as previously suggested (12). Experimentally testable predictions can be made that discriminate these models. For example, if dGTP must be the first nucleotide to add to a repositioned product to observe dGTP-dependent repeat addition processivity, then altering the template residue dictating this nucleotide addition specificity should abrogate the dGTP-dependence of repeat addition processivity. On the other hand, if the dGTP interaction required for repeat addition processivity is independent of the template, any template sequence change allowing wild-type levels of product synthesis to the template 5′-end, rebinding of the product 3′-end at the template 3′-end and product-anchor site interaction should allow processive repeat addition stimulated by dGTP. Our data indicate that dGTP-stimulated repeat addition processivity is accomplished in a manner different from envisioned by either model above.

**MATERIALS AND METHODS**

Recombinant Telomerase Production—T. thermophila telomerase RNA expression constructs were derived from pT7159 (16) by site-specific mutagenesis. Pairs of complementary mutagenic oligonucleotides were used in a linear amplification reaction with Phi polymerase and double-stranded DNA templates (17). All telomerase RNA expression constructs were sequenced to confirm the presence of only the intended change. To express telomerase RNAs with the wild-type RNA 3′-end, plasmids were digested with FokI and reprecipitated after organic extraction. T. thermophila TERT was expressed from the plasmid p33CITE (11). Equal masses of telomerase RNA-encoding plasmid and telomerase protein-encoding plasmid were combined for coupled transcription/translation in rabbit reticulocyte lysate (Promega TNT). This produces roughly equimolar amounts of telomerase RNAs and TERT (about 10 nM). For the determination of relative activity in Table I, expression reactions were analyzed by Northern blot to verify comparable levels of telomerase RNAs and by immunoblot to verify comparable levels of TERT. For the experiment shown in Fig. 7, N-terminal hemagglutinin epitope-tagged TERT was coexpressed with telomerase RNA, purified by binding to immobilized HA antibody and eluted with peptide.

**Telomerase Activity Assay**—Activity assays were performed under conditions similar to those previously described for recombinant Tetrahymena telomerase (11). Typically, 2 μl or less of reticulocyte lysate expression reaction was diluted to 10 μl in T2MG (20 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 10% glycerol, ≤ 2.5 mM dithiothreitol) and used in a 20-μl final assay volume in 1× assay buffer (50 mM Tris acetate, pH 8.0, 2 mM MgCl₂, 5 mM β-mercaptoethanol, 10 mM spermidine). Nucleotides were included as indicated in the figure legends. DNA oligonucleotide primers were used at 1 μM final concentration, unless otherwise specified. Products were incubated at 30 °C for 1 h unless otherwise specified. Product DNA was extracted with phenol/chloroform/isoamyl alcohol, precipitated with ammonium acetate, and resolved by denaturing gel electrophoresis. In some experiments, a radiolabeled 80-nt oligonucleotide was added before extraction and precipitation as an internal control for sample recovery (see Fig. 7).

**Quantitation of Repeat Addition Processivity**—Quantitation of product intensity was done by phosphorimager (Fuji). Relative product intensities were converted to relative molar amounts of product by normalizing for the number of incorporated dGTPs. The molar amount of second repeat addition product divided by the sum of the first and second repeat addition products is a measure of repeat addition processivity. For example, for primers TG, TTG, repeat addition processivity is determined as molar amount (primer +12)/(sum of primer +6 and primer +12). This calculation does not include the small amount of product lost by elongation with additional repeats, which is difficult to quantitate reliably and would not substantially affect the values obtained. Similar values for repeat addition processivity were obtained comparing first and second repeat addition products, second and third repeat addition products, or third and fourth repeat addition products. Also, repeat addition processivity values were similar when calculated from a 15 or 60 min reaction, indicating that use of these reaction times did not limit repeat addition processivity.

**RESULTS**

The Influence of Template Position 48 on Repeat Addition Processivity—Tetrahymena telomerase repeat addition processivity was assayed by elongation of an excess of the DNA primer (TG)₈TTG in the presence of [³²P]dGTP and dTTP. This primer binds with its 3′-end at the template 3′-end (Fig. 1A). Products with lengths of primer +1 to primer +6 can be synthesized in the addition of the first repeat (Fig. 1A). Because primer is in greater than 1,000-fold excess over telomerase enzyme and telomerase products (data not shown), products longer than primer +6 derive from processive repeat addition. In 0.6 μM dGTP, which is sufficient for high nucleotide addition processivity within a repeat, recombinant Tetrahymena telomerase added predominantly one repeat to each bound (TG)₈TTG primer (Fig. 1B, WT lane 0.6 μM dGTP). Because of differences in absolute dGTP concentration in different shipments of [³²P]dGTP, some variation in the absolute repeat addition processivity attained with 0.6 μM dGTP assays containing only radiolabeled dGTP stock was observed (compare Figs. 1–4 and 6). With increasing dGTP concentration, increasing repeat addition processivity allowed the elongation of some primers by many repeats before dissociation (Fig. 1B, WT lanes 2.6–40 μM dGTP). Because unlabeled dGTP was added to dilute the radiolabeled dGTP stock for dGTP concentrations greater than 0.6 μM, dGTP specific activity was reduced as dGTP concentration increased, and product intensity decreased accordingly. However, if product intensity was adjusted for specific activity, the total amount of product was similar at all dGTP concentrations. In this study, we quantitated repeat addition processivity by determining the amount of first repeat addition product that was extended by addition of a second repeat on a molar basis (see “Materials and Methods”). For the wild-type enzyme reactions shown in Fig. 1, repeat addition processivity increased from 3.1% at 0.6 μM dGTP to 22% at 40 μM dGTP.

The first nucleotide added to a product repositioned for processive repeat synthesis is templated by the RNA residue C-48. To assess the influence of this template position on repeat addition processivity, we created expression constructs encoding the altered telomerase RNAs C48G, C48U, and C48A. Coexpression of these RNAs with TERT produced recombinant telomerase enzymes, which still bound the (TG)₈TTG primer at the 5′-end (Fig. 1B). The C48N telomerases were assayed with [³²P]dGTP, dTTP, and dATP (C48G) or dTTP (C48U). Initial elongation of the primer (TG)₈TTG occurred by addition of dCTP (C48G), dATP (C48U), dTTP (C48A), or dGTP (WT). In 0.6 μM dGTP, repeat addition processivity was low for all enzymes (Fig. 1B). If dGTP concentration was increased to 2.6, 10, or 40 μM, the repeat addition processivity of all C48N enzymes was stimulated (Fig. 1B). The maximum attainable repeat addition processivity varied with template sequence (Table I). For example, the C48U and C48A enzymes had lower maximal repeat addition processivity than the C48G and WT
FIG. 1. The repeat addition processivity of all C48N enzymes is similarly dependent on the concentration of dGTP. A, template region of the *T. thermophila* telomerase RNA spans positions C-43 to A-51. The primer (TG)\textsubscript{8}TTG binds at the template 3'-end and is elongated by up to 6 nt in first repeat addition. Repositioning of a product 3'-end from the template 5'-to 3'-end can allow second repeat addition. B, each C48N enzyme was assayed with the primer (TG)\textsubscript{8}TTG. Reactions contained 0.6 μM [\textsuperscript{32}P]dGTP and extra unlabeled dGTP to obtain 0.6–40 μM total dGTP. All reactions contained 200 μM dTTP, reactions with C48G also contained 200 μM dCTP, and reactions with C48U also contained 200 μM dCTP, and reactions with C48U also contained 200 μM dATP. The migration of primer extended by addition of various numbers of nucleotides is indicated. Note that the first radiolabeled product of the C48N enzymes other than wild-type has a length of primer + 2 because of the initial addition of an unlabeled nucleotide. Repeat addition processivity as a function of dGTP concentration is indicated (values at 0.6 μM dGTP for C48U and C48A are not possible to calculate accurately because of the poly(dG) product background). A shorter exposure of C48U lanes and a longer exposure of C48A lanes are also shown below.

### Table I

**Summary of quantitation**

| Telomerase RNA | maximum repeat addition processivity\(^a\) | repeat addition processivity relative to WT | overall activity relative to WT\(^b\) |
|----------------|---------------------------------------------|-------------------------------------------|-------------------------------------|
| Wild-type (WT) | 22 % | 100 | 100 |
| C48G           | 14 % | 64 | 17 |
| C48U           | 3.4 % | 18 | 760 |
| C48A           | 5.8 % | 27 | 14 |
| C48G9G         | N.D.\(^c\) | N.D. | ≤10 |
| 3C             | 15 | 68 | 130 |
| 5C             | N.D. | N.D. | ≤10 |
| 5' + 2U (-dTTP) | 16 | 73 | ≤10 |
| 5' + 2U (+dTTP) | N.D. | N.D. | ≤10 |

\(^a\) This value is molar amount (primer + 12)/(sum of primer + 6 and primer + 12) except for 3C, for which the value is molar amount (primer + 10)/(sum of primer + 5 and primer + 10). Values were calculated from assays with 40 μM dGTP.

\(^b\) Values were calculated from assays with 0.6 μM dGTP because this condition generated the smallest size range of product DNA. Values were similar when calculated from assays at other dGTP concentrations and from other sets of assays (data not shown). In the assay set quantitated here, C48U RNA expression was 2-fold more than that of the wild-type RNA. Because the relative percentage of each RNA that is active is unknown, values in the table were not normalized.

\(^c\) N.D. indicates not reliably determined due to particularly low repeat addition processivity.

The concentration of dGTP required to stimulate maximal repeat addition processivity varied with template sequence, the concentration of dGTP required to stimulate maximal repeat addition processivity was similar for all C48N enzymes. We conclude that the dGTP concentration dependence of repeat addition processivity does not depend on the sequence of template position 48.

Next, dGTP concentration was fixed at 0.6 μM whereas the concentration of the nucleotide cognate to template position 48 was varied from 0 to 125 μM (Fig. 2). In no case did increasing the concentration of a dNTP other than dGTP stimulate repeat addition processivity, even if nucleotide concentration was raised to the threshold at which telomerase activity was inhibited nonspecifically (data not shown). In the absence of the dNTP cognate to template position 48, none of the altered templates allowed substantial incorporation of [\textsuperscript{32}P]dGTP (Fig. 2, lanes 0 μM dNTP). This establishes that dGTP was not misincorporated by the C48G, C48U, and C48A enzymes at template position 48, which could have allowed a dGTP-stimulated repeat addition processivity still requiring the first nucleotide addition of dGTP. Trace product synthesis with C48G enzyme in the absence of dCTP may derive from incorporation of dTTP at the template position 48G, whereas the primer-sized product of C48A synthesized in the absence of dTTP derives from cleavage and readdition of the primer 3'G, templated by either 43C or 49C. From the assays in Fig. 2, we conclude that the first nucleotide added to a template-repositioned product does not determine the nucleotide dependence of repeat addition processivity.

In the experiments described above, a surprising lack of correlation was observed between the relative activity level and the relative repeat addition processivity directed by different template sequences. Based on product intensity, the C48U enzyme (3.4 and 5.8% versus 14 and 22%). This is likely to originate from the reduced nucleotide addition processivity of the C48U and C48A enzymes, evident in the enhanced accumulation of mid-template dissociation products. This reduced nucleotide addition processivity is expected, based on the reduced stability of an A-U or T-A product-template hybrid compared with the wild-type G-C. Although maximal repeat addition processivity varied with template sequence, the concentration of dGTP required to stimulate maximal repeat addition processivity was similar for all C48N enzymes. We conclude that the dGTP concentration dependence of repeat addition processivity does not depend on the sequence of template position 48.
enzyme outperformed the wild-type enzyme, which in turn outperformed the C48G and C48A enzymes (Table I). This rank order differs from that of repeat and nucleotide addition processivities, in which the WT and C48G enzymes outperformed the C48A and C48U enzymes. Unlike the differences in processivity, the differences in overall activity cannot be explained by predicted alterations in the stability of the product-template hybrid.

**Nucleotide Structural Requirements for Stimulation of Repeat Addition Processivity**—To investigate which features of the dGTP nucleotide were important for its ability to stimulate repeat addition processivity, we tested whether other purine nucleotides could enhance repeat addition processivity in reactions with 0.6 μM dGTP. Addition of the ribonucleotide triphosphate GTP or deoxyinosine triphosphate (dITP) at concentrations up to 125 μM failed to enhance repeat addition processivity (Fig. 3A). GTP differs from dGTP by the presence of a ribose C-2’-hydroxyl group, whereas dITP differs from dGTP by the lack of the amino group on base C-2. Thus, addition of a C-2’ hydroxyl or loss of the C-2 amino group both prevent dGTP from stimulating repeat addition processivity. GTP is incorporated poorly by *Tetrahymena* telomerase (18), and neither GTP nor dITP inhibited incorporation of [32P]dGTP under the conditions assayed (Fig. 3A).

We also examined 7-deaza-dGTP, which differs from dGTP by carbon substitution of the base N-7. Addition of 7-deaza-dGTP to reactions with 0.6 μM dGTP had three consequences: stimulation of repeat addition processivity, inhibition of [32P]dGTP incorporation, and alteration of the product profile to promote accumulation of products with lengths of primer +3, +9, etc. (Fig. 3A). Each of these effects of 7-deaza-dGTP was competed by addition of extra dGTP, with the ratio of products altered as a reflection of the ratio of dGTP to 7-deaza-dGTP in the reaction (Fig. 3A, right). The altered product profile was also observed in assays with [32P]dTTP and 7-deaza-dGTP alone, without any dGTP (data not shown). In reactions with 0.6 μM [32P]dGTP without dTTP, 7-deaza-dGTP and dCTP both stimulated synthesis of a polynucleotide ladder (Fig. 3B). These results suggest that 7-deaza-dGTP is efficiently incorporated by telomerase into both telomeric repeats and a polynucleotide ladder, but that 7-deaza-dGTP incorporation into telomeric repeats affects the product profile. The change in product pattern is likely to reflect a change in the stability of the product-template hybrid. Independent of its possible impact on hybrid stability, however, the repeat addition processivity stimulated by 7-deaza-dGTP indicates that the dGTP nitrogen at base position 7 is not an essential property of a processivity-stimulatory nucleotide.

We next examined the role of the dGTP triphosphate group. Surprisingly, addition of 2.6, 10, or 40 μM of dGMP, dGDP, or dGTP to assays with 0.6 μM [32P]dGTP stimulated repeat addition processivity (Fig. 4A). The maximal repeat addition processivity obtained with titration of the concentration of each of these three nucleotides was strikingly similar, as was the concentration of nucleotide required to obtain it. In contrast, addition of up to 250 μM deoxyguanosine did not stimulate repeat addition processivity (Fig. 4B). Although dGMP and dGDP cannot be incorporated, they inhibited product synthesis (Fig. 4, A and B). This inhibition was eliminated by addition of extra dGTP (data not shown). In contrast, deoxyguanosine did not inhibit product synthesis (Fig. 4B). We conclude that the nucleotide-stimulating repeat addition processivity requires only a monophosphate group. In addition, we note that all nucleotides that stimulate repeat addition processivity reduce dGTP incorporation, whether or not they can be incorporated themselves. This suggests that the processivity-stimulatory dGTP (or dGTP analog) is binding in the active site with a specificity that parallels that of dGTP binding for nucleotide incorporation (see “Discussion”).

**Template Requirements for Repeat Addition Processivity**—In addition to template position 48 and a substrate dNTP, the active site elongating a repositioned product would be strongly influenced by the terminal product-template base pair. To investigate the role of this base pair, composed of a product G and template C in the wild-type enzyme, we created expression constructs encoding C4349U and C4349G telomerase RNAs. It was necessary to coordinately change both positions C-43 and C-49 to obtain processive repeat addition, because a product 5'-end (Fig. 5A). Telomerases with wild-type, C4349U, or C4349G templates were assayed with the primers (TG)6TTG, (TG)6TTA, or (TG)6TTC, respectively. The C4349U enzyme assayed with (TG)6TTC had robust activity (Table I) and enough nucleotide addition processivity for substantial complete first repeat synthesis (Fig. 5B). In contrast with the C48N enzymes, however, the addition of up to 40 μM dGTP stimulated only a trace amount of second repeat addition (Fig. 5B). Various concentra-
tions of dCTP (data not shown) or 100 μM dGMP (Fig. 5C) did not significantly stimulate the repeat addition processivity of the C4349G enzyme.

There were several possible explanations for the inhibited repeat addition processivity of the C4349G enzyme. As a first possibility, product elongated to the template 5'-end could have failed to dissociate, preventing additional product elongation. In fact, low product turnover limits budding yeast telomerase to a single round of repeat addition per primer binding event in vitro (19). One simple method to investigate failed dissociation was to assay a time course of product synthesis, because only if product turnover occurs could product continue to accumulate with time. Product accumulation occurred continuously over the entire time course of a standard assay for both the wild-type and C4349G enzymes (Fig. 5C), suggesting that the inhibition of C4349G enzyme repeat addition processivity does not

FIG. 3. Structural variants of dGTP have different abilities to stimulate repeat addition processivity. Wild-type enzyme was assayed with the primer (TG)₈TTG. The migration of primer extended by addition of various numbers of nucleotides is indicated. A, reactions contained 0.6 μM [³²P]dGTP, 200 μM dTTP, and concentrations of the indicated nucleotides from 0–125 μM. Some reactions also contained 10 μM extra unlabeled dGTP as indicated. A longer exposure of the right 6 lanes is also shown below. B, reactions contained 0.6 μM [³²P]dGTP, no dTTP, and concentrations of the indicated nucleotides from 1–100 μM.

FIG. 4. Only one phosphate group of dGTP is required to stimulate repeat addition processivity. Wild-type enzyme was assayed with the primer (TG)₈TTG. The migration of primer extended by addition of various numbers of nucleotides is indicated. A, reactions contained 0.6 μM [³²P]dGTP, 400 μM dTTP, and concentrations of the indicated nucleotides from 0–40 μM. Repeat addition processivity as a function of dGTP concentration is indicated. B, reactions contained 1.2 μM [³²P]dGTP, 400 μM dTTP, and concentrations of dGMP or deoxyguanosine (dG) from 0–250 μM.
Fig. 5. Repeat addition processivity requires more than wild-type levels of overall activity, product-template hybrid stability, and product-anchor site interaction. A, C4349G enzyme binds the primer (TG)$_8$TTC at the 5′-end of the template. The first repeat addition product can reposition from the template 5′- to 3′-end. B, the C4349G enzyme was assayed with the primer (TG)$_8$TTC. Reactions contained 0.6 μM [32P]dGTP, extra unlabeled dGTP to obtain 0.6–40 μM total dGTP, 200 μM dCTP, and 200 μM dTTP. The migration of primer extended by addition of various numbers of nucleotides is indicated. A longer exposure is also shown at right. C, the wild-type and C4349G enzymes were assayed with the primers (TG)$_8$TTG and (TG)$_8$TTC, respectively. Reactions contained 3.0 μM dGTP, 200 μM dTTP, and for C4349G also 200 μM dCTP. Aliquots of a single reaction were stopped after 12, 24, or 48 min. Separate assays contained an additional 100 μM dGMP. The migration of primer extended by addition of various numbers of nucleotides is indicated. D, the wild-type enzyme was assayed with the indicated primers at 1 μM concentration for either a 15- or 60-min reaction time. Reactions contained 2.6 μM dGTP and 400 μM dTTP. The migration of primer extended by addition of various numbers of nucleotides is indicated.

We also investigated the role of a particular length of template and/or product repeat in promoting repeat addition processivity. We created telomerase RNA expression constructs that truncated or extended the template, encoding either a 5-nt T$_6$G$_4$ repeat (3C) or a 7-nt T$_7$G$_5$ repeat (5C) instead of the wild-type 6-nt T$_6$G$_4$ repeat (Fig. 6, A and B). Similar template substitutions have been reconstituted by recombinant RNA with endogenous, micrococcal nuclease-treated Tetrahymena proteins in vitro (20) or by expression of a recombinant Tetrahymena telomerase RNA gene in vitro (21). Our 3C enzyme, composed of recombinant TERK and telomerase RNA, had strong telomerase activity (Table I) and the expected product ladder of 5-nt rather than 6-nt periodicity (Fig. 6A). In addition, the 3C enzyme catalyzed nucleolytic cleavage of the primer (TG)$_8$TTC to generate substantial radiolabeled product with length of primer +0. Maximal repeat addition processivity was within 2-fold of that of the wild-type enzyme (15 versus 24% in parallel reactions at 40 μM dGTP; Fig. 6A) and required a similar dGTP concentration. In vitro reconstitution of a 3C template with endogenous proteins also produced a telomerase enzyme with some repeat addition processivity (20).

In contrast, the 5C recombinant enzyme had both reduced activity and dramatically reduced repeat addition processivity (Table I, Fig. 6B). A 5C template reconstituted with endogenous proteins in vitro had similarly little if any repeat addition processivity (20), whereas the repeat addition processivity of 5C enzyme reconstituted in vivo was inhibited but still substantial (21). The very low repeat addition processivity that could be detected for our recombinant 5C enzyme still required a dGTP concentration over micromolar. Overall activity and maximal repeat addition processivity were not improved by using primers capable of more initial hybridization with the template (data not shown). Also, if the first repeat addition product of the 5C enzyme was assayed as a primer for the wild-type enzyme, it was as efficiently and processively elongated or product template dissociation, rebinding of the product 3′-end or product interaction at the substrate anchor site. This suggests that these activities are not sufficient for repeat addition processivity.

derive from a failure of product turnover. As a second possibility, the repositioned product 3′-end could have formed a weaker hybrid with the template 3′-end. However, the primers (TG)$_8$TTG and (TG)$_8$TTC were elongated at similar concentrations by the wild-type and C4349G enzymes, respectively (data not shown). This suggests that the product-template hybrid formed at the C4349G template 3′-end was sufficiently stable to allow repeat addition processivity.

As a third possibility, the part of the repositioned product sequence that does not bind the template could have formed a weaker interaction at the substrate anchor site, resulting in product dissociation. To address this possibility, primers with anchor site sequences corresponding to the products of first repeat addition with wild-type and C4349G templates ((TG)$_8$TTGGGGTTG and (TG)$_8$TTTCGGGTTG) were assayed with wild-type enzyme. The (TG)$_8$TTGGGGTTG primer promoted synthesis of slightly more product than (TG)$_8$TTTCGGGTTG, but the two primers were elongated at similar K$_{cat}$ and similar repeat addition processivities (Fig. 5D and data not shown). The lower maximal activity with (TG)$_8$TTGGGGTTG likely derives from inhibitory binding of some primer across the entire template, rather than at the template 3′-end. This mode of binding would also account for the enhanced nucleolytic cleavage of this primer, evident in the accumulation of radiolabeled product with length of primer +0 (Fig. 5D). Cleavage occurs preferentially for substrates aligned at the template 5′-end (6). Unlike the (TG)$_8$TTGGGGTTG primer, unlike the (TG)$_8$TTTCGGGTTG primer, can align with the template 5′-end without template-primer mismatch. Considering all the data for the C4349G enzyme, we conclude that its low repeat addition processivity does not reflect the compromise of any molecular event previously known to be required for repeat addition processivity: product-template dissociation, rebinding of the product 3′-end at the template 5′-end or product interaction at the substrate anchor site. This suggests that these activities are not sufficient for repeat addition processivity.
gated as a primer with wild-type or 3C template product se-
quence (Fig. 6C and data not shown). These results suggest
that the inhibition of 5C enzyme activity and repeat addition
processivity does not derive from formation of a weaker prod-
uct-template hybrid or a weaker product-anchor site interac-
tion. Furthermore, product accumulation continued over the
time course of a standard 5C enzyme reaction, demonstrating
that the inhibition of repeat addition processivity does not
derive from a failure of product turnover (data not shown). We
conclude that as for the C4349G enzyme, no molecular event
known to be required for repeat addition processivity compro-
misses the repeat addition processivity of the 5C enzyme.

The 5C template change altered both template length and
product repeat length. To determine whether altered template
length alone inhibited overall activity or maximal repeat addi-
tion processivity, we created a telomerase RNA expression
construct with two uridines inserted immediately adjacent to
the last template residue of the wild-type RNA (5'-2U; Fig.
6D). In assays containing dATP, the primer (TG)₈TTG was
elongated by 8 nt in first repeat addition. In assays lacking
dATP, however, the wild-type 6-nt repeat was synthesized.
Assayed with or without dATP, the overall activity of the
5'-2U enzyme was reduced compared with the wild-type en-
zyme (Table I). Repeat addition processivity was negligible in
reactions with dATP (Fig. 6D), responsible at least in part to
the inability of the first repeat addition product to rebind at the
template 3'-end. In reactions without dATP, however, repeat
addition processivity was within 2-fold of that of the wild-type
enzyme (Table I, Fig. 6D). We conclude that increased template
length inhibits overall activity independent of any change in
product length or repeat addition processivity. However, in-
creased template length alone, without a change in product
length, does not affect dGTP-dependent repeat addition pro-
cessivity.

**Anchor Site Independence of Repeat Addition Processivity—**
The inhibited repeat addition processivity of C4349G and 5C
enzymes, above shown to be independent of any previously
known processivity requirement, prompted us to examine
whether the dGTP-dependent repeat addition processivity of
recombinant *Tetrahymena* telomerase was accomplished by a
fundamentally different mechanism than the dGTP-independ-
ent repeat addition processivity of the endogenous enzyme. To
test this, we compared the concentration dependence and re-
peat addition processivity of primer elongation for a set of
primers with the same 3'-end but different 5'-lengths or se-
quences. Because each primer forms the same 3'-interaction
with the template, differences in a primer Km for elongation or
in elongation repeat addition processivity derive from differ-
ences in template-independent enzyme-product interactions.
For endogenous *Tetrahymena* telomerase, increasing the
length of a primer from 6 to 10 nt substantially increases
primer binding affinity because of anchor site interactions (9, 10).
For the recombinant enzyme, increasing the length of a
primer from 6 to 10 nt also slightly increased apparent primer
binding affinity (Fig. 7 and data not shown).

For endogenous *Tetrahymena* telomerase, increasing the
length of a primer from 6 to 10 nt increases the repeat addition
processivity of elongation dramatically (9, 10). In contrast, for
the recombinant enzyme, even a 6-nnt primer had maximal repeat addition processivity (Fig. 7). If anything, increasing primer length slightly decreased repeat addition processivity for primers composed of perfect telomeric repeats (16% with T2G4, 12% with G4T2G4, 11% with (T2G4)3). Processive elongation of all primers, independent of length or sequence, required greater than micromolar concentrations of dGTP (data not shown). We conclude that the repeat addition processivity of recombinant Tetrahymena telomerase does not depend on an anchor site interaction similar to that described for the endogenous enzyme. Instead, there is a distinct, dGTP-dependent mechanism for repeat addition processivity that involves enzyme interaction with nascent product DNA (Fig. 8). It is the grip of this nascent product DNA binding site on product released from the template, rather than the grip of an anchor site on more 5' product sequence, that allows dGTP-dependent repeat addition processivity. The modest extent of repeat addition processivity gained by the dGTP-dependent mechanism may not be evident in assays of endogenous Tetrahymena telomerase with substrates that are capable of anchor site interaction. However, using short primer DNAs with the endogenous enzyme, a stimulation of repeat addition processivity at greater than micromolar dGTP concentrations was indeed observed (data not shown). The quantitative impact of dGTP stimulation is difficult to assess for the endogenous enzyme, because any product that gains a second repeat will efficiently engage the dGTP-independent repeat addition processivity mechanism.

**DISCUSSION**

**Template- and Nucleotide-dependence of Repeat Addition Processivity**—We have defined molecular requirements for the repeat addition processivity of a recombinant Tetrahymena telomerase RNP composed of telomerase RNA and TERT. Changes in the template sequence that reduce nucleotide addition processivity, including the substitutions C48U and C48A, also reduce maximal repeat addition processivity. Apart from its influence on nucleotide addition processivity, however, the identity of the base at template position 48 is not important for the basic mechanism of repeat addition processivity or for establishing the dependence of repeat addition processivity on dGTP. Insertion of extra telomerase RNA nucleotides immediately 5' of the template also does not inhibit repeat addition processivity, indicating that there is not a strict spacing required between the template and the TERT binding site 5' of the template (22). In contrast, despite maintaining high nucleotide addition processivity, the template sequence alterations C4349G and 5C inhibit repeat addition processivity almost
completely. The RNA residues affected by these substitutions may be critical for repeat addition processivity directly or it may be that the products of these templates are the basis of the inhibition observed. Because the C4349G and 5C substitutions allow complete first repeat addition but inhibit repeat addition processivity, these substitutions may affect the same molecular event as the processivity-stimulatory binding of dGTP.

In addition to template requirements, we also investigated nucleotide structural requirements for repeat addition processivity. We found that deoxyguanosine monophosphate promotes as much repeat addition processivity as deoxyguanosine triphosphate and has a similar $K_m$ for stimulation of repeat addition processivity as well. This suggests that triphosphate hydrolysis is not required to stimulate repeat addition processivity. In addition, we found that the C-2 amino group of dGTP is essential but N-7 is not. Purine ribonucleotides including GTP (shown above) and XTP (data not shown) do not stimulate repeat addition processivity.

Our data argue strongly for binding of the processivity-stimulatory dGTP at the active site but leave open the nature of the processivity-determining molecular event. It is possible that dGTP either promotes the interaction of nascent product with enzyme (Fig. 8, step 1) or promotes a subsequent conformational change required to reposition the product 3′-end (Fig. 8, step 2). We have shown that the dGTP-dependence of repeat addition processivity does not derive from its role as the first nucleotide added to a repositioned product. Our results also indicate that an influence of dGTP on the dissociation of product-template hybrid is unlikely, because similar amounts of product are synthesized at all dGTP concentrations, and substantial product turnover occurs even when repeat addition is not processive. Thus, nascent product binding or product 3′-end repositioning are more likely targets of stimulation by dGTP. Additional insight will require a more direct investigation of the product interaction sites in recombinant telomerase.

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