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Negative regulation of yeast telomerase activity through an interaction with an upstream region of the DNA primer

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ABSTRACT

A number of published studies indicate that telomerase may interact with oligonucleotide primers in a bipartite manner, with the 3′-end of the primer positioned at the catalytic site of the enzyme and a more 5′ region of the primer binding to a second or 'anchor' site of the enzyme. We systematically investigated the effects of mutations in the DNA primer on overall binding and polymerization by yeast telomerase. Our studies indicate that there is sequence-specific interaction between telomerase and a substantial region of the DNA primer. Mutations in the 3′-most positions of the primer reduced polymerization, yet had little effect on overall binding affinity. In contrast, mutations around the –20 position reduced binding affinity but had no effect on polymerization. Most strikingly, mutations centered around the –12 position of the DNA primer reduced overall binding affinity but dramatically enhanced primer extension, as well as primer cleavage. This finding suggests that reduced interaction with the –12 region of the DNA primer can facilitate a step in the catalytic region of yeast telomerase that leads to greater polymerization. A tripartite model of interaction between primer and telomerase is proposed to account for the distinct effects of mutations in different regions of the DNA primer.

INTRODUCTION

Telomerase is a ribonucleoprotein (RNP) that is responsible for synthesis of the dG-rich strand of telomere terminal repeats (1–3). The enzyme was initially identified in *Tetrahymena thermophila*, a ciliated protozoan whose life cycle involves formation of a large number of telomeres. The original *in vitro* assay was based on the ability of telomerase to extend telomere-like oligodeoxynucleotides in the presence of dGTP and dTTP (4). The enzyme was subsequently found to be an unusual reverse transcriptase containing an integral RNA component, a small segment of which acts as the template for synthesis of the dGT-rich strand of telomeric repeats (5). Multiple repeats can be added to the input primer following initial binding, despite the minimal number of repeat units in the RNA template, suggesting that the enzyme must undergo a translocation step during processive DNA synthesis (6). Telomerase can also cleave the input primer under certain conditions (7–9), a property that is shared by a number of DNA and RNA polymerases.

Telomerase activity has been detected in a wide range of organisms, including protozoa (10), yeast (8, 11–13), mouse (14), *Xenopus* (15) and human (16). Genes encoding the RNA component of the enzyme complex have been cloned for many telomerases, such as that of yeast and human (17,18). Recently some of the polypeptide components of telomerase were cloned. In particular, a protein named p123 in *Euplotes aediculatus* and a homologous protein encoded by *EST2* in yeast were shown to be the catalytic components of the respective telomerases (19,20); these two polypeptides exhibit significant homology to other reverse transcriptases and mutations that alter Est2p residues that are conserved among reverse transcriptases abolish telomerase activity *in vitro* and telomerase function *in vivo*. In addition, two polypeptides, p80 and p95, that co-purify with *Tetrahymena* telomerase have been cloned and been shown to interact with telomerase RNA and the DNA primer respectively (21). Mouse and human homologs of p80 have also been identified and been shown to associate with the respective telomerases (22,23). These recent developments should greatly facilitate structure–function analysis of telomerase.

A number of experiments indicate that telomerase may interact with oligonucleotide primers in a bipartite manner, with the 3′-end of the primer positioned at the catalytic site of the enzyme and a 5′-region binding to a second or 'anchor' site of the enzyme. First, the processivity of *Tetrahymena* telomerase was greatly influenced by the length of the oligonucleotide: primers <10 nt were extended by only one repeat whereas longer primers were processively elongated (7,24). This suggests that binding to the upstream region ('anchor' site) of the primer prevents dissociation of the enzyme during translocation, which is needed for synthesis of long products. Second, a non-telomeric primer that was a poor substrate for telomerase elongation is a much better substrate when telomeric repeats were added to its 5′-end, again suggestive of an upstream interaction site in the enzyme (25–27). Third, the efficiency of primer utilization was altered when the 3′-sequence

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of the primer was held constant and the 5′-sequence was varied (24). Direct evidence for a telomerase 'anchor' site came from a study where the –20 to –22 region of a DNA primer was shown to crosslink to a telomerase protein and the telomerase RNA from *Euplotes aediculatus* (28).

To further analyze primer–telomerase interactions we tested the effects of a large number of mutations (defined as deviations from canonical yeast telomere sequence) in the oligonucleotide primer on its ability to bind and to be extended by yeast telomerase. Our studies indicate that there is sequence-specific interaction between yeast telomerase and an extended region of the DNA primer. Mutations in different regions of the DNA primer have very distinct effects on binding and polymerization. A tripartite model of interaction between primer and yeast telomerase is proposed to account for these observations. A particularly interesting aspect of the model is that telomerase activity can be negatively regulated through an interaction with the –12 region of the DNA primer.

MATERIALS AND METHODS

Yeast strains, media and buffers

The haploid *Saccharomyces cerevisiae* strain DG338 was used throughout. The cells were grown in YPD medium (2% glucose, 1.0% Yeast Extract, 2% Bacto-peptone). The following protease inhibitors were included in all buffers: 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 2 µg/ml pepstatin A and 1 µg/ml leupeptin. Buffer TMG-15 contains 15% glycerol, 10 mM Tris–HCl, pH 8.0, 1.2 mM magnesium chloride, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM DTT. Buffer TMG-10 is identical to TMG-15 except that glycerol was included at 10%. Buffer TMG-10(500) etc. denotes buffer TMG-10 plus the concentration of sodium acetate (mM) specified by the number in parentheses.

Preparation of yeast telomerase

Yeast telomerase was prepared according to Cohn and Blackburn (8) with only slight modifications. Four liters of *S.cerevisiae* cultures were grown to late log phase, harvested and washed once with ice-cold water. Cell pellets were resuspended in an equal volume of TMG-15, mixed with a volume of glass beads equal to the total suspension volume and lysed by vortexing at maximum speed. Thirty seconds of vortexing was alternated with 30 s cooling in an ice–water bath for a total of 25 min. Cell lysates were collected and cleared by two successive centrifugations: first in a GSA rotor (Sorvall) at 8000 r.p.m. for 10 min and then in a T-865 rotor (Sorvall) at 32 000 r.p.m. for 1 h.

Telomerase activity was partially purified by passage through a DEAE-agarose (BioRad) column. A 5 ml DEAE column was equilibrated in TMG-10(0). The extract (20 ml) was loaded directly onto the column. The column was washed successively with 10 ml TMG-10(300) and 10 ml TMG-10(550) and the activity eluted with 10 ml TMG-10(900). Fractions were collected and used directly in telomerase assays.

Primer extension assays for *S.cerevisiae* telomerase

A typical telomerase reaction was carried out in 30 µl containing the following: 10 mM Tris–HCl, pH 8.0, 2 mM magnesium acetate, ~300 mM sodium acetate (contributed by the protein fraction), 1 mM spermidine, 1 mM DTT, 5% glycerol (contributed by the protein fraction), 50 µM dTTP, 20 µCi [α-^{32}P]dGTP (3000 Ci/mmol), 5 µM primer oligodeoxynucleotides and ~15 µl column fractions. Following incubation at 30°C for 1–1.5 h the reaction was stopped by adding 80 µl 10 mM Tris–HCl, pH 8.0, 20 mM EDTA, 0.1 mg/ml RNase A. Following incubation at room temperature for 10 min the products were further digested with 100 µl solution containing 10 mM Tris–HCl, pH 8.0, 0.5% SDS and 0.3 mg/ml proteinase K at 37°C for 20 min. The digested reaction products were precipitated with the addition of 750 µl absolute ethanol and 100 µl 7.5 M ammonium acetate containing 1 µg yeast tRNA and analyzed on a 15% TBE–urea polyacrylamide gel. Signals were quantified using a PhosphorImager system (Molecular Dynamics). For quantification of activity the signals from all labeled and RNase-sensitive products (including those that are shorter than the input primer) are summed.

Oligonucleotide labeling and gel mobility shift assay

DNA primers used for gel mobility shift experiments were radiolabeled with T4 polynucleotide kinase and purified through a Nick Column (Pharmacia). The gel mobility shift assay is based on a previously described protocol (29). Partially purified telomerase was incubated with 5 nM (0.4 ng) labeled primer in 16 µl buffer containing 10 mM Tris–HCl, pH 8.0, 2 mM $MgCl_2$, 12% glycerol and 50 ng poly(dI·dC). Where indicated, cold competitor oligonucleotides were added before addition of telomerase. Binding was carried out at 4°C for 20 min and complex formation monitored by electrophoresis through a 4% polyacrylamide gel with running buffer containing 25 mM Tris–HCl, pH 8.3, 190 mM glycine, 5 mM $MgCl_2$ and 1 mM EDTA. The gel was cast in running buffer supplemented with 10% (v/v) glycerol and 0.5 mM DTT.

RESULTS

A TLC1-directed yeast telomerase activity

One of us has previously reported a processive telomerase-like activity from *S.cerevisiae* that is TLC1-dependent (12,30). However, subsequent studies suggest that the fraction used may be contaminated with a second polymerase activity (data not shown). We therefore experimented with other published protocols for yeast telomerase derivation and found that a high salt eluate from a DEAE column, as reported by Cohn and Blackburn (8), has a robust RNase-sensitive polymerization activity that is TLC1-dependent (data not shown). Preparation of the fraction from a strain harboring point mutations in the TLC1 template region yielded an activity that has the expected property for nucleotide utilization. The TLC1-1 mutation substituted a GG dinucleotide for AC such that the enzyme can incorporate dCMP in addition to dGMP and dTMP (Fig. 1A; 17). To facilitate incorporation of dCMP by the mutant enzyme a primer that ends in a dGGG trinucleotide was used because this trinucleotide is immediately upstream of the expected dC residue. As shown in Figure 1, the enzyme preparation from the TLC1-1 strain supported extension of up to 3 nt in the presence of dCTP and dTTP (presumably the dCCT trinucleotide) and >7 nt in the presence of dCTP, dGTP and dTTP (the triangles in Fig. 1 denote the positions of 'primer + 1'). A small amount of polymerization is also observed when labeled dGTP and unlabeled dTTP are used, presumably as a consequence of alternative alignment and/or primer cleavage. In contrast, the activity from a wild-type strain does not incorporate a significant amount of dCMP, even

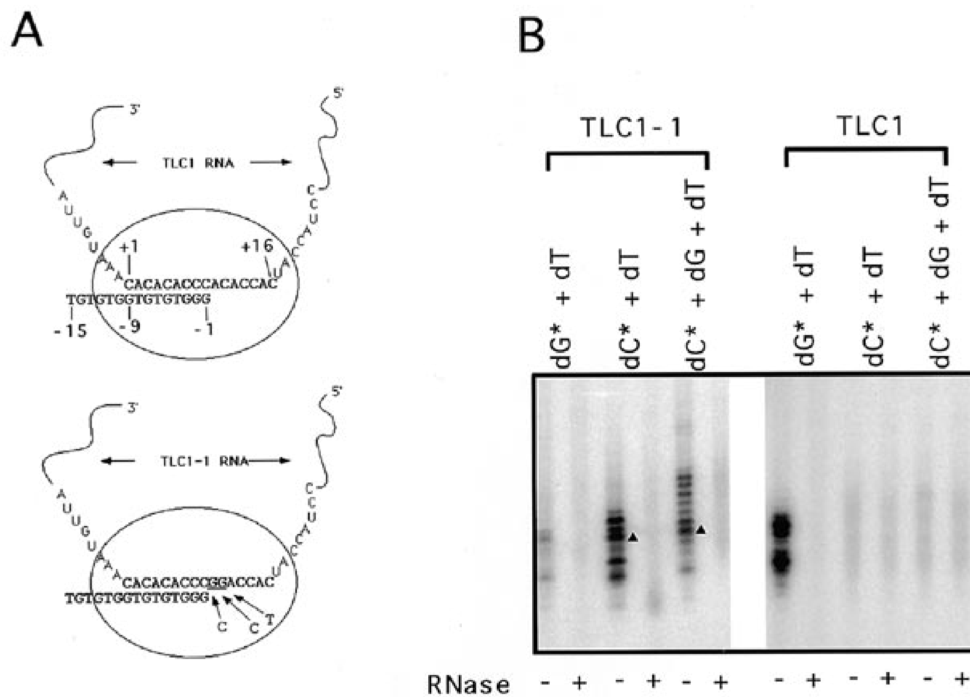

Figure 1. Yeast telomerase bearing mutations in the RNA template has altered nucleotide usage. (**A**) A schematic illustration of the alignment between yeast telomerase (with either a wild-type or a mutant RNA) and oligonucleotide primer. The positions of the ribonucleotides within the TLC1 template region are numbered from +1 to +16 in the 3′→5′ direction. The positions of the deoxynucleotides in the DNA primer are numbered from –1 down, with –1 being the 3′-most deoxynucleotide. (**B**) Primer extension assays for yeast telomerase prepared from a wild-type (TLC1) or a mutant (TLC1-1) strain were carried out using a 16 nt oligo (TGTCTGGGTGTCTGGG) as primer and different combinations of labeled (*) and unlabeled deoxynucleotides as substrates. The filled triangles indicate the position of the 'primer + 1' product. Reactions were carried out using fractions that have (+) or have not (–) been pretreated with RNase A.

in the presence of both dGTP and dTTP. Consistent with earlier reports, we find that telomerase activity derived in this fashion acts non-processively *in vitro* and is not stimulated by ATP (data not shown). Since this fraction appears largely devoid of other contaminating activity, we have used it exclusively for subsequent assays.

Mutations in an upstream region of the DNA primer stimulate primer extension

To determine the nature and significance of the interaction between telomerase and different parts of the primer we tested the effects of defined sequence changes in the primer on its utilization by telomerase. As expected, point mutations in the 3′-most positions (–1 to –3) reduced the level of incorporation (Fig. 2A), consistent with the role of these nucleotides in aligning with the RNA template. In contrast, point mutations anywhere in the –4 to –14 region of a 15 nt primer consistently stimulated primer utilization (Fig. 2B and Tables 1 and 2). The effect was maximal with mutations around the –12 region (~20-fold; Fig. 2B and Table 2) and diminishes with mutations upstream or downstream. In addition to enhanced polymerization, the relative proportion of the products that were shorter than the input primer also increased (data not shown). Because >95% of the input primer remained undegraded (data not shown), the nuclease activity appeared to be tightly associated with yeast telomerase. It has previously been reported that primer/template mismatch can enhance primer cleavage by yeast telomerase (31). However, this series of primers, which end in TGGG, were designed to align with only 9 bases of the template. Thus the effects of mutations upstream of the –9 position cannot be accounted for by mismatch. Instead, altered interaction with a second site of the enzyme is likely to be responsible.

The nature of the mutation does not appear to greatly influence the degree of stimulation. For example, changing the dG residues at the –7 position to either dA, dC or dT resulted in comparable enhancement in primer utilization (5-fold for a G→T change and 12-fold for a G→A change). The same was true for mutations at the –12 position: changing the dG base to any one of the other three bases resulted in an ~20-fold stimulation of polymerization.

The time course of the reactions indicates that preferential utilization of mutant primers was due to an increase in the rate of DNA synthesis, rather than simply an increase in the overall extent of synthesis. As shown in Figure 2D, the rate of DNA synthesis for a wild-type primer (TEL15) was significantly greater than a mutant primer [TEL15(–12)G→T] over a 20 min period. The reaction was essentially complete after 20 min and further incubation had little effect on the final signal (data not shown).

Because of the simultaneous increase in products that are both longer and shorter than the input primer, we tested the hypothesis that increased cleavage followed by elongation was responsible for the increase in the amount of longer products. A very efficient mutant primer TEL15(–12)G→C and the wild-type primer TEL15 were tested for telomerase utilization in the presence of dTTP and ddGTP. If nuclease cleavage is obligatory for extension, then one would expect little or no product in the mutant reaction that is longer than the starting primer. However, as shown in Figure 2E, the TEL15(–12)G→C reaction yielded a product at the 'primer + 2' position that was ~20 times more intense than the equivalent band in the TEL15 reaction, consistent with direct extension of the input

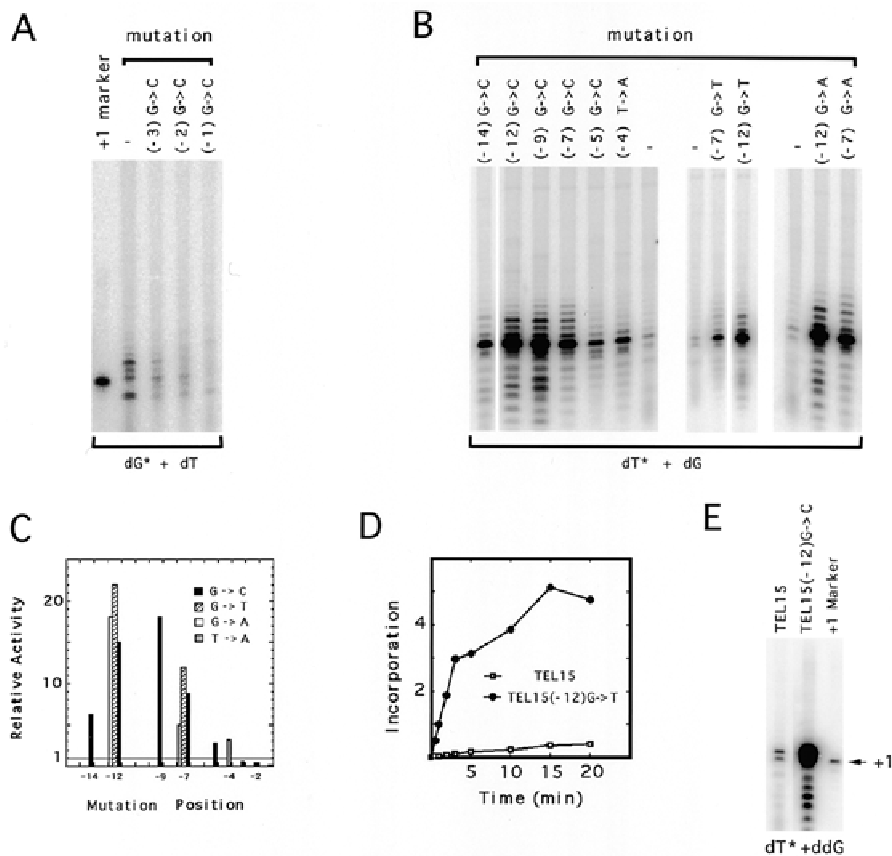

Figure 2. DNA primers with mutations in the upstream region are preferentially polymerized by yeast telomerase. (**A**) Primer extension assays for yeast telomerase were carried out using 5 µM 15 nt primers (series 1 in Table 1), labeled dGTP and unlabeled dTTP. The position and nature of the mutation within each primer are indicated at the top of the figure. All mutations are G→C changes. The leftmost lane shows TEL15 labeled with cordycepin and terminal transferase, which co-migrates with the 'primer + 1' product. (**B**) Primer extensions assays for yeast telomerase were carried out using 5 µM 15 nt primers (series 1 in Table 1), labeled dTTP and unlabeled dGTP. The position and nature of the mutation within each primer are indicated at the top of the figure. (**C**) The relative activities of the primers in extension assays are plotted against the positions of the mutations. The wild-type primer is arbitrarily defined as having an activity of 1. (**D**) Plots for the time course of telomerase reaction for the TEL15 and TEL15(–12)G→T primers. (**E**) Primer extension assays for yeast telomerase were carried out using a combination of labeled dTTP and unlabeled dideoxyGTP. The primers used are indicated at the top of the figure. The rightmost lane shows TEL15 labeled with cordycepin and terminal transferase, which co-migrates with the 'primer + 1' product.

primer even in the case of the mutant primer. Thus mutations in the upstream region appear to stimulate two separate reactions by telomerase, direct polymerization and primer cleavage coupled with extension. Furthermore, the pattern of the short products in the dT + ddG reactions indicates that extension occurred at multiple positions, consistent with progressive exonucleolytic cleavage or endonucleolytic cleavage at multiple positions.

The stimulatory effects of primer mutations were not dependent upon particular reaction parameters. For example, when labeled dGTP was used in combination with unlabeled dTTP the same degree of stimulation was observed (data not shown). Varying the sodium acetate (from 30 to 300 mM) and magnesium (from 1 to 5 mM) concentration also had little effect on the magnitude of stimulation (data not shown).

The stimulatory effect of upstream mutations is not specific for a particular template position or for a particular primer length

All of the primers used in the previous section are designed to align with TLC1 RNA such that the 3′-end residue is positioned at the +9 position of the template (see Fig. 1A for an illustration). To rule out the possibility that the stimulatory effect is specific for this type of primer we tested oligonucleotides with a different 3′-sequence that should support formation of a 12 base hybrid (Table 1, series 3). As shown in Figure 3A and Table 2, mutations in the upstream region of these 19 nt oligonucleotides again stimulated polymerization by telomerase, with the maximal effect observed for a '–12 & –13' double mutant [TEL19(–12)G→C(–13)T→A].

The consequence of varying the length of the primer on the stimulatory effects of mutations was also investigated. When a set of 24 nt primers (with identical 3′-ends to the 15 nt primers) were assayed for extension by telomerase a primer that bears two point mutations at the –12 and –13 position [TEL24(–12)T→A-(–13)G→C] was again found to support a much higher level of polymerization and cleavage, whereas one that bears two point mutations at the –20 and –21 positions [TEL24(–20)T→A-(–21)G→C] had the same activity as the wild-type primer (Fig. 3B and Table 2). In these reactions an RNase-insensitive band that migrated slightly faster than the input primer can be seen (indicated by ◆ in Fig. 3B). This was probably due to the activity of an unrelated enzyme.

The results presented in Figures 2 and 3 together point to the functional importance of the interaction between yeast telomerase

Table 1. Primers

Primer	Sequence
Series #1 (15nt)	
TEL15	TGTGTGGTGTGTGGG
TEL15(-1)G→C	TGTGTGGTGTGTGGC
TEL15(-2)G→C	TGTGTGGTGTGTGCG
TEL15(-3)G→C	TGTGTGGTGTGTCGG
TEL15(-4)T→A	TGTGTGGTGTGAGGG
TEL15(-5)G→C	TGTGTGGTGTCTGGG
TEL15(-7)G→C	TGTGTGGTCTGTGGG
TEL15(-7)G→T	TGTGTGGTTTGTGGG
TEL15(-7)G→A	TGTGTGGTATGTGGG
TEL15(-9)G→C	TGTGTGCTGTGTGGG
TEL15(-12)G→C	TGTCTGGTGTGTGGG
TEL15(-12)G→T	TGTTTGGTGTGTGGG
TEL15(-12)G→A	TGTATGGTGTGTGGG
TEL15(-14)G→C	TCTGTGGTGTGTGGG
Series #2 (24nt)	
TEL24	TGTGTGGGTGTGTGGGTGTGTGGG
TEL24(-12)T→A(-13)G→C	TGTGTGGGTGTCAGGGTGTGTGGG
TEL24(-20)T→A(-21)G→C	TGTCAGGGTGTGTGGGTGTGTGGG
Series #3 (19nt)	
TEL19	TGTGTGGGTGTGTGGGTGT
TEL19(-6)G→C(-7)T→A	TGTGTGGGTGTGACGGTGT
TEL19(-9)T→A(-10)G→C	TGTGTGGGTCAGTGGGTGT
TEL19(-10)G→C	TGTGTGGGTCTGTGGGTGT
TEL19(-12)G→C(-13)T→A	TGTGTGACTGTGTGGGTGT
TEL19(-16)G→C	TGTCTGGGTGTGTGGGTGT
TEL19(-18)G→C	TCTGTGGGTGTGTGGGTGT
Series #4 (short)	
TEL11	TGGTGTGTGGG
TEL9	GTGTGTGGG
TEL6	TGTGGG

Four different series of primers are used for extension and binding assays. Positions that deviate from canonical yeast telomere sequences are underlined. All primers are named according to their lengths and mutations. For example, TEL15(–3)G→C is a 15 nt primer carrying a G→C mutation at the –3 position.

Table 2. Activites of the primers in binding and extension assays

Primer	Mutation	Activity^	Molar ratio required for 1/2 competition#
series 1 (15nt)			
TEL15	None	1	1 : 1
TEL15(-1)G→C	-1	0.21	1.9 : 1
TEL15(-2)G→C	-2	0.47	1.9 : 1
TEL15(-3)G→C	-3	0.58	4.3 : 1
TEL15(-4)T→A	-4	3.2	8 : 1
TEL15(-5)G→C	-5	2.8	45 : 1
TEL15(-7)G→C	-7	8.8	5.3 : 1
TEL15(-7)G→T	-7	5.0	6.9 : 1
TEL15(-7)G→A	-7	12	6.7 : 1
TEL15(-9)G→C	-9	18	5.9 : 1
TEL15(-12)G→C	-12	15	72 : 1
TEL15(-12)G→T	-12	18	75 : 1
TEL15(-12)G→A	-12	22	75 : 1
TEL15(-14)G→C	-14	6.2	2.7 : 1
series 2 (24nt)			
TEL24	None	1	1 : 1
TEL24(-12)T→A(-13)G→C	-12, -13	17	3.7 : 1
TEL24(-20)T→A(-21)G→C	-20, -21	1	3.2 : 1
series 3 (19nt)			
TEL19	None	1	N.D.±
TEL19(-6)G→C(-7)T→A	-6 & -7	1	N.D.
TEL19(-9)T→A(-10)G→C	-9 & -10	2.8	N.D.
TEL19(-10)G→C	-10	1.2	N.D.
TEL19(-12)G→C(-13)T→A	-12 & -13	10.8	N.D.
TEL19(-16)G→C	-16	1	N.D.
TEL19(-18)G→C	-18	1.5	N.D.
series 4 (short)			
TEL11	deletion (11mer)	12*	200 : 1
TEL9	deletion (9mer)	4.3*	170 : 1
TEL6	deletion (6mer)	2.0*	340 : 1

^The relative activity of each primer was determined in standard primer extension assays using labeled dTTP and unlabeled dGTP. For each series of oligonucleotides the activity was normalized against the wild-type primer.
*Normalized against TEL15
#Molar ratio of unlabeled oligonucleotide to labeled TEL15 required for 50% competition of the signal observed for the telomerase–TEL15 complex.
±Not determined

and around the –12 region of the DNA primer. In addition, two observations indicate that this important DNA target site is measured from the 3′-end of the primer: (i) mutations around the –12 region consistently exhibited the greatest effects regardless of the 3′-end position or the total length of the primer; (ii) the TEL19(–16)G→C and TEL15(–12)G→C primers, which have the same 7 nt sequence (with one G→C point mutation) at their 5′-end but differ in the location of the mutation relative to the 3′-end, supported very different degrees of stimulation (no stimulation for the former and 20-fold for the latter).

Short primers lacking the upstream interaction site support greater polymerization by yeast telomerase

Because there is no reason to suspect that yeast telomerase will bind more strongly to all the mutant primers, we hypothesized that there is a negative correlation between the strength of the upstream interaction (centered around the –12 position of the primer) and activity of the primers in standard polymerization assays. If this is indeed the case, shorter primers that lack this upstream interaction region may better support polymerization. Primers with the same 3′-end sequence but ranging in size from 6 to 15 nt were synthesized and tested in polymerization assays (Fig. 4 and Tables 1 and 2). Consistent with our hypothesis, an 11 nt primer (TEL11) was 12 times and a 9 nt primer (TEL9) 4 times more active than TEL15. The 6 nt primer (TEL6) was only twice as active, possibly because it did not efficiently or stably form a hybrid with telomerase RNA.

Mutations throughout the upstream region of the DNA primer diminish its overall affinity for yeast telomerase

To obtain direct evidence for reduced binding between telomerase and short or mutant primers we measured the overall affinity of yeast telomerase for the various primers in gel mobility shift experiments. When the telomerase-containing fraction was incubated with a labeled single-stranded telomere oligonucleotide (TEL15) and then electrophoresed through a non-denaturing polyacrylamide gel a number of distinct complexes could be visualized. Only the complex with the lowest mobility, which migrated as an ~1 kb double-stranded DNA, was abolished when the fraction was pretreated with RNase A (Fig. 5A, marked with a dot). Furthermore, addition of an RNase inhibitor prior to RNase A preserved the low mobility complex (RNase + Inh, Fig. 5A), whereas addition of the inhibitor following RNase A treatment failed to restore complex formation [RNase (Inh), Fig. 5A]. A number of observations indicate that this RNase-sensitive binding activity is specific for the G-rich strand of yeast telomeres. First, the C-rich strand of yeast telomere on its own did not support complex formation (data not shown). Second, converting the single-stranded probe to a double-stranded oligonucleotide abolished complex formation (Fig. 5B,

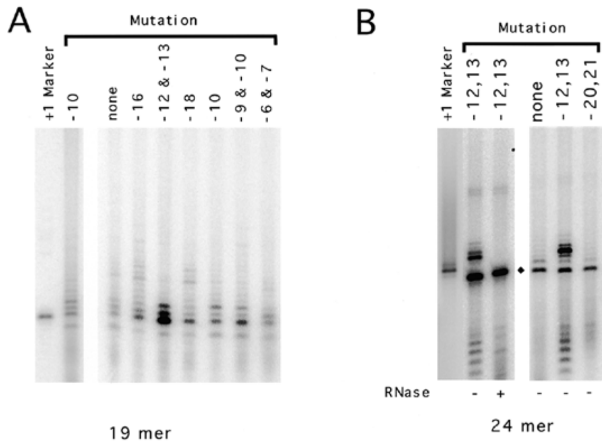

Figure 3. The stimulatory effects of the mutations in the DNA primers are not dependent upon the 3′-end sequence or the total length of the primer. (**A**) Primer extension assays for yeast telomerase were carried out using a series of 19 nt primers (series 3 in Table 1), labeled dGTP and unlabeled dTTP. The positions of the mutations within the DNA primers are indicated at the top of the figure. The leftmost lane shows TEL19 labeled with cordycepin and terminal transferase, which co-migrates with the 'primer + 1' product. (**B**) Primer extension assays for yeast telomerase were carried out using a series of 24 nt primers (series 2 in Table 1), labeled dGTP and unlabeled dTTP. The positions of the mutations within the DNA primers are indicated at the top of the figure. The leftmost lane shows TEL24 labeled with cordycepin and terminal transferase, which co-migrates with the 'primer + 1' product. An RNase-insensitive band (indicated by ◆) can be visualized in this set of assays.

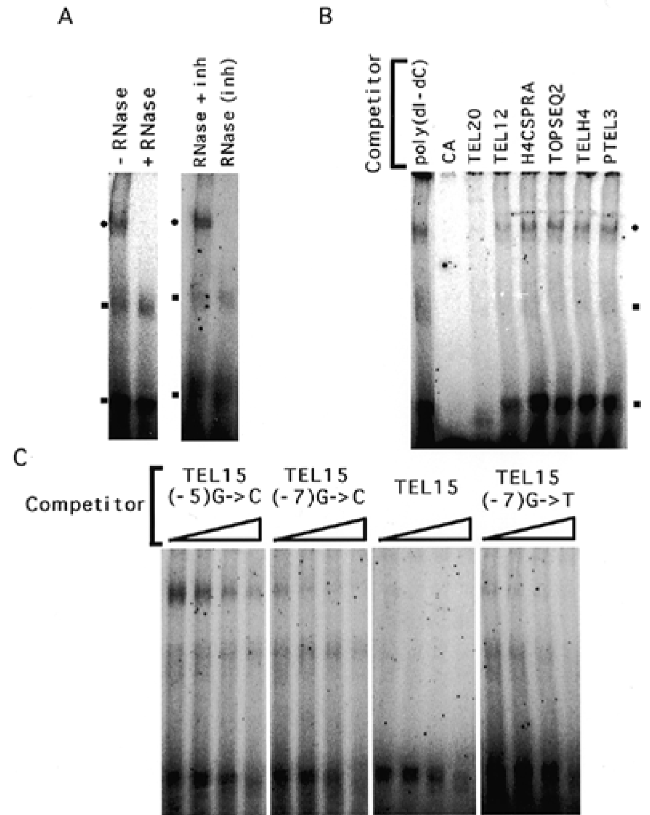

Figure 5. The affinity of the DNA primer for yeast telomerase is reduced by point mutations in the primer. (**A**) Gel mobility shift assays were carried out using labeled TEL15 oligonucleotide as probe and partially purified yeast telomerase. As controls the telomerase fraction was pre-incubated with 10 ng RNase A at 20°C for 10 min (+ RNase), with 10 ng RNase A and 2 U of Prime-Inhibitor (5′→3′ Inc., Boulder, CO) (RNase + inh) or with RNase A for 10 min followed by the addition of Prime-Inhibitor [RNase (inh)]. Three different complexes (marked by either a dot or a square) can be visualized. Only the slowest migrating complex (marked by a dot) is abolished by pretreatment of the fraction with RNase A. (**B**) Competition gel mobility shift assays were carried out using labeled TEL15 as probe and either poly(dI·dC) DNA (at 100-fold excess by weight) or various oligonucleotides (at 100-fold excess by molar concentration) as competitors. The sequences of the oligonucleotides were: CA, CCCACACACCACACA; TEL20, TGTGTGGGTGTGTGGG; TEL12, TGTCTGGGTGTCTGGG; H4CSPRA, TTTTAATGATGGAA; TOPSEQ2, ACAGATTCGTTTGGAAAGAG; TELH4, CTTCCCATGGTAATTAAAA; PTEL3, GGTTACAGGTTACAGGTT. The CA oligonucleotide is complementary to TEL15 and is expected to convert the probe to a double-stranded oligonucleotide. (**C**) Gel mobility shift assays were carried out using labeled TEL15 as probe, 50 ng poly(dI·dC) as carrier and various oligonucleotides as competitors (indicated at the top of each panel). For each set of competition assays the molar excesses of competitor to probe used were 5, 15, 45 and 135 (left to right).

Figure 4. The optimal length of the DNA primer for yeast telomerase in primer extension assays is ~11 nt. Primer extension assays for yeast telomerase were carried out using primers with the same 3′-end sequence but different lengths. The lengths and the names of the primers used are indicated at the top of the figure.

lane CA). Finally, adding a number of non-telomeric oligonucleotides or telomeric oligonucleotides from other species had little effect on the pattern of mobility shift (Fig. 5B, lanes H4CSPRA, TOPSEQ2, TELH4 and PTEL3). These observations, together with the fact that the RNase-sensitive complex co-migrates with TLC1 RNA (data not shown), strongly suggest that the low mobility complex is due to telomerase.

A series of competition experiments were carried out to determine the relative affinities of the various oligonucleotides used for polymerization (Fig. 5C and Table 2). Our results show that there is sequence-specific interaction between yeast telomerase and an extended region of the DNA primer (from –1 to at least –21): most of the mutant primers exhibited reduced affinity for yeast telomerase. Interestingly, mutations in the 3′-most positions (–1 to –3) had the mildest effects on affinity (≤4-fold), despite their role in aligning with the RNA template. Thus the hybrid may be short or transient and may contribute little to stable binding. In

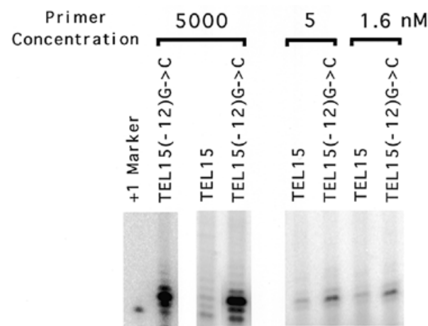

Figure 6. Lowering the primer concentration reduced the degree of stimulation by mutations. Primer extension assays for yeast telomerase were carried out using three different concentrations of either TEL15 or TEL15(–12)G→C as primers (indicated at the top of the figure). The leftmost lane shows TEL15 labeled with cordycepin and terminal transferase, which co-migrates with the 'primer + 1' product.

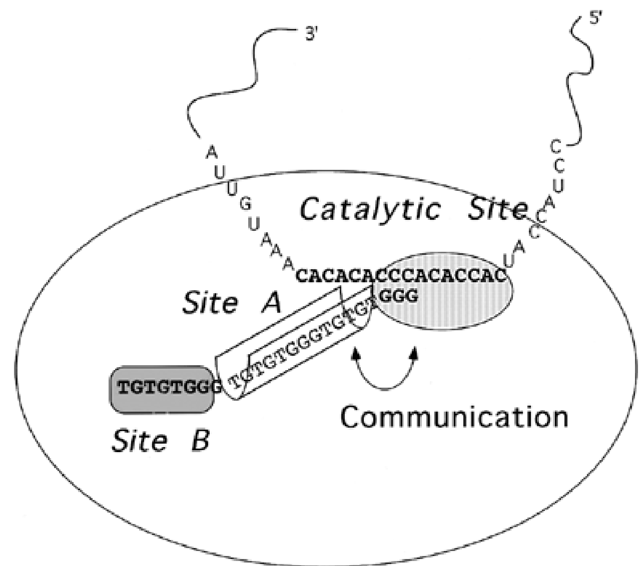

Figure 7. A tripartite model of interaction between yeast telomerase and primer oligonucleotide. See Discussion for explanation.

contrast, mutations in the –4 to –14 region consistently had a greater negative effect on affinity, with the '–12' mutants [TEL15(–12)G→C, TEL15(–12)G→T and TEL15(–12)G→A among the 15 nt primers] showing the weakest binding to telomerase (~70-fold reduction in affinity; Table 2). Short primers lacking the upstream sites also consistently bound less tightly to yeast telomerase. Thus, while there is not a strict negative correlation between binding and polymerization, our results suggest that in general weakened interaction with the –12 region of the primer facilitates polymerization by yeast telomerase.

Even though mutations in the –16 to –21 region of the primer have little effect on polymerization, they can also lower the overall affinity of the primer for the enzyme, by ~3- to 4-fold [compare TEL24(–20)T→A(–21)G→C with TEL24]. This implies the existence of a functionally distinct interaction between telomerase and the far upstream region of the primer, one that does not affect polymerization but may serve other purposes, such as an 'anchor' site function (28).

The magnitude of stimulation of DNA synthesis by upstream mutations in the primer is reduced at low primer concentrations

Since telomerase must capture the DNA primer before polymerization can take place, it may seem surprising that the lowered affinity of the mutant primers does not result in reduced DNA synthesis. However, in standard polymerization assays the primer is present at micromolar concentrations, orders of magnitude higher than the estimated K_d. Thus an effect of lower binding affinity is likely to be masked. However, such an effect should be revealed when the primers are tested at concentrations approaching the dissociation constants (~3 nM; data not shown). This is indeed the case: when the polymerization assays were done with 1.6–5 nM primer the effect of a '–12' mutation is only 3-fold, rather than the 20-fold seen at micromolar concentrations (Fig. 6).

DISCUSSION

Our results suggest that the interaction between yeast telomerase and oligonucleotide primer is tripartite, as illustrated in Figure 7. The 3′-end of the primer forms a DNA–RNA hybrid with the TLC1 telomerase RNA. The 5′-region of the primer can be functionally subdivided into two parts: the –4 to –14 region of the primer interacts with one site of the enzyme (site A in Fig. 7), while the –16 to –21 region of the primer interacts with another site (site B in Fig. 7). The location of the target of site B in our model suggests that it may correspond to the previously characterized 'anchor' site in ciliate telomerase (28). Both site A and site B interactions appear to contribute to the overall binding energy between telomerase and primer. However, only site A interaction influences polymerization at the catalytic site; loss of site A interaction facilitates polymerization. Possibly, conformational alteration of the enzyme at site A can facilitate an important conformational or chemical step at the catalytic site. Loss of site A interaction also appears to facilitate primer cleavage coupled with extension. How these two types of stimulation are related is not known.

The precise region of the primer that interacts with site A is unclear. Mutations in the –4 to –9 region of the primer have qualitatively similar effects on binding and polymerization as mutations in the –10 to –14 region, suggesting that both regions are targets for site A binding. However, the –4 to –9 region of the primer can also potentially form a hybrid with telomerase RNA and be juxtaposed to the active site. Thus mutations in the –4 to –9 region of the primer can conceivably stimulate polymerization by a distinct mechanism. Indeed, an earlier study on yeast telomerase concluded that mismatches between the –4 to –6 region of the primer and template RNA promote slippage synthesis and primer cleavage (31). However, given the current lack of information on the precise extent of RNA–DNA hybrid formation during binding and polymerization, the results in the earlier study can also be interpreted in terms of altered interaction between site A of the enzyme and the upstream region of the primer.

The subunits of the telomerase that encompass site A or site B in our model are not known. Given the close functional connection between the catalytic site and site A it is tempting to suggest that the latter is also formed by Est2p and TLC1, the catalytic subunits. In addition, if the experimental results from *Euplotes aediculatus* can be directly extrapolated to yeast then

site B may also be formed by Est2p and TLC1; the –20 region of the DNA primer (corresponding to site B in our model) can be crosslinked to p123 (the Est2p homolog) and the RNA component of *Euplotes* telomerase. However, a role for other subunits of telomerase (e.g. Est1p; 11,13,32) in formation of site A or site B cannot yet be ruled out.

Previous studies with telomerase from other species also strongly support an interaction between the enzyme and upstream region of the primer as mentioned in the Introduction. In particular, a thorough study of the kinetics of polymerization by *Tetrahymena* telomerase revealed profound sequence-specific effects of the –7 to –12 region of the DNA primer (24). Furthermore, the effects of primer length on polymerization by *Tetrahymena* telomerase are very similar to the yeast enzyme, with the highest V_{max} achieved by a 10 nt primer and lower values by longer and shorter primers. Likewise, for *Oxytrica* telomerase the rate of synthesis is higher with an 8 nt primer than with a 16 nt primer (33). Thus the functional consequence of this upstream interaction in the case of ciliate telomerase may also be similar to yeast.

Very little is known about the mechanisms by which telomerase activity is regulated *in vivo*. Our results on the inhibitory effects of site A interaction on telomerase activity raise the possibility of an intriguing mode of regulation. Perhaps *in vivo* telomerase activity is repressed by tight interaction between part of the telomeric tail and site A of the enzyme. Activation of telomerase activity would then involve a step that weakens this interaction, possibly through binding of or modification by another factor.

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REFERENCES

1 Blackburn,E.H. and Greider,C.W. (1995) *Telomeres*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2 Zakian,V. (1995) *Science*, **270**, 1601–1607.
3 Blackburn,E.H. (1991) *Nature*, **350**, 569–573.
4 Greider,C.W. and Blackburn,E.H. (1985) *Cell*, **43**, 405–413.
5 Greider,C.W. and Blackburn,E.H. (1989) *Nature*, **337**, 331–337.
6 Greider,C.W. (1991) *Mol. Cell. Biol*., **11**, 4572–4580.
7 Collins,K. and Greider,C.W. (1993) *Genes Dev*., **7**, 1364–1376.
8 Cohn,M. and Blackburn,E.H. (1995) *Science*, **269**, 396–400.
9 Melek,M., Greene,E.C. and Shippen,D.E. (1996) *Mol. Cell. Biol*., **16**, 3437–3445.
10 Blackburn,E.H. (1992) *Annu. Rev. Biochem*., **61**, 113–129.
11 Lin,J.-J. and Zakian,V. (1995) *Cell*, **81**, 1127–1135.
12 Lue,N.F. and Wang,J. (1995) *J. Biol. Chem*., **270**, 21453–21456.
13 Steiner,B.R., Hidaka,K. and Futcher,B. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 2817–2821.
14 Prowse,K.R., Avilion,A.A. and Greider,C.W. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 1493–1497.
15 Mantell,L.L. and Greider,C.W. (1994) *EMBO J*., **13**, 3211–3213.
16 Morin,G. (1989) *Cell*, **59**, 521–529.
17 Singer,M.S. and Gottschling,D.E. (1994) *Science*, **266**, 404–409.
18 Feng,J., Funk,W.D., Wang,S.-S., Weinrich,S.L., Avillion,A.A., Chiu,C.-P., Adams,R.R., Chang,E., Allsopp,R.C., Yu,J., Le,S., West,M.D., Harley,C.B., Andrews,W.H., Greider,C.W. and Villeponteau,B. (1995) *Science*, **269**, 1236–1241.
19 Lendvay,T.S., Morris,D.K., Sah,J., Balasubramanian,B. and Lundblad,V. (1996) *Genetics*, **144**, 1399–1412.
20 Lingner,J., Hughes,T.R., Shevchenko,A., Mann,M., Lundblad,V. and Cech,T.R. (1997) *Science*, **276**, 561–567.
21 Collins,K., Kobayashi,R. and Greider,C.W. (1995) *Cell*, **81**, 677–686.
22 Harrington,L., McPhail,T., Mar,V., Zhou,W., Oulton,R., Amgen EST Program, Bass,M.B., Arruda,I. and Robinson,M.O. (1997) *Science*, **275**, 973–977.
23 Nakayama,J.-I., Saito,M., Nakamura,H., Matsuura,A. and Ishikawa,F. (1997) *Cell*, **88**, 875–884.
24 Lee,M. and Blackburn,E.H. (1993) *Mol. Cell. Biol*., **13**, 6586–6599.
25 Harrington,L.A. and Greider,C.W. (1991) *Nature*, **353**, 451–454.
26 Morin,G.B. (1991) *Nature*, **353**, 454–456.
27 Wang,H. and Blackburn,E.H. (1997) *EMBO J*., **16**, 866–879.
28 Hammond,P.W., Lively,T.N. and Cech,T.R. (1997) *Mol. Cell. Biol*., **17**, 296–308.
29 Horikoshi,M., Wang,C.K., Fujii,H., Cromlish,J.A., Weil,P.A. and Roeder,R.G. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4843–4847.
30 Nugent,C.I., Hughes,T.R., Lue,N.F. and Lundblad,V. (1996) *Science* **274**, 249–252.
31 Prescott,J. and Blackburn,E.H. (1997) *Genes Dev*., **11**, 528–540.
32 Virta-Pearlman,V., Morris,D.K. and Lundblad,V. (1996) *Genes Dev*., **10**, 3094–3104.
33 Zahler,A.M., Williamson,J.R., Cech,T.R. and Prescott,D.M. (1991) *Nature*, **350**, 718–720.