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A novel specificity for the primer–template pairing requirement in *Tetrahymena* telomerase

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Telomerase is a specialized reverse transcriptase with a built-in RNA template. Base pairing between the templating domain of telomerase RNA and a telomeric DNA primer is normally a characteristic of elongation of telomeric DNA. Here we demonstrate the mechanism by which *Tetrahymena* telomerase bypasses a requirement for template–primer pairing in order to add telomeric DNA *de novo* to completely non-telomeric DNA primers. We show that this reaction initiates by copying the template residue at the 3′ boundary of the telomerase RNA template sequence. Unexpectedly, as the RNA template moves through the telomerase catalytic center, the number of required potential Watson–Crick base pairs between RNA template and DNA primer increases from zero to five. We propose that this unprecedented position specificity of a base pairing potential requirement in a polymerase underlies the chromosome healing mechanism of telomerase, and reflects constraints inherent in an internal template.
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Introduction

Biosynthesis of telomeric DNA at the ends of linear eukaryotic chromosomes involves telomerase, a ribonucleoprotein (RNP) reverse transcriptase. Telomere synthesis takes place in two different situations: first, the ends of eukaryotic chromosomes undergo continuous replenishment via addition of telomeric repeat units to the telomeric sequence already present at the termini, thereby overcoming the gradual shortening that occurs in the absence of telomerase (reviewed in Zakian, 1995); second, making a new telomere (chromosomal healing) involves broken chromosomal ends lacking telomeric repeat tracts regaining their stability through *de novo* addition of telomeric repeats by telomerase (Yu and Blackburn, 1991; Kramer and Haber, 1993; Blackburn, 1995).

Telomerase contains an essential reverse transcriptase-like protein subunit (Lingner *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). Unlike viral reverse transcriptases, the template of telomerase is confined to a short region of the telomerase RNA, which is an intrinsic part of the active telomerase RNP complex (Greider and Blackburn, 1989; Greider, 1995). Much of the remaining telomerase RNA outside the templating domain is apparently buried and inaccessible in the telomerase RNP (Greider and Blackburn, 1989; Zaug and Cech, 1995). The templating domain of *Tetrahymena* telomerase RNA consists of the nine telomere-complementary nucleotides 3′-aaCCCCAAC-5′ (nucleotides 51–43 in the telomerase RNA). The seven nucleotides indicated in upper case (49–43) are known to template synthesis of repeats of the telomeric DNA sequence G_4T_2. Position 49 is the 3′ most residue that can be copied (Gilley and Blackburn, 1996). In the replenishment reaction, via which telomerase was identified initially, 'alignment' of telomeric sequence primers involves canonical Watson–Crick base pairing between the 3′ end of the primer and the template region and dictates the initiation site on the template (Blackburn, 1992; Greider, 1995). Binding of telomeric primers is stabilized further by interactions of the 5′ region of the telomeric or G-rich primer with a second site, the anchor site, of telomerase (Collins and Greider, 1993; Lee *et al.*, 1993). The anchor site for telomeric DNA is located on both a protein subunit and a region of the telomerase RNA outside the templating domain (Hammond *et al.*, 1997). However, broken chromosomal ends generally contain little or no telomeric sequences, precluding base pairing between primer and template and stable anchor site binding at the initiation step of healing (Yao *et al.*, 1987; Pologe and Ravetch, 1988; Wilkie *et al.*, 1990; Yu and Blackburn, 1991; Scherf *et al.*, 1992; Lamb *et al.*, 1993; Fan and Yao, 1996; Melek and Shippen, 1996; Wicky *et al.*, 1996).

Tetrahymena thermophila telomerase *in vitro* can add telomeric G_4T_2 repeats efficiently *de novo* onto completely non-telomeric DNA substrates (chromosomal healing), initiating with the same d(GGGGT...) sequence as *in vivo*. Such addition relies on primer length-dependent interactions between the single-stranded non-telomeric DNA substrate and the telomerase RNP (Wang and Blackburn, 1997). Melek *et al.* (1996) reported that *Euplotes* telomerase does not elongate non-telomeric primers completely *in vitro*. However, when a telomeric sequence was added to create a chimeric primer with a 3′ non-telomeric end and a 5′ telomeric region, 'default' telomere addition occurred, with G_4T_4 being the first added sequence, a reaction possibly facilitated by a developmentally regulated factor other than telomerase (Melek *et al.*, 1996; Bednenko *et al.*, 1997). However, in neither system was it unequivocally shown which template C residues were copied into the initially added G4 sequence.

Here we used mutant *Tetrahymena* telomerases, containing base changes in the telomerase RNA template, to localize the initiation of *de novo* telomere addition to non-telomeric primers to the 3′ boundary of the template, i.e. to nt 49. In addition, we report that, surprisingly, productive polymerization requires potential pairing between the 3′

end of the primer and template in a manner that is strongly dependent on the template position being copied. This requirement progressively increases as the RNA template moves through the catalytic site, from no duplex required when copying position 49, up to a 5 bp potential duplex when copying positions 44 and 43. This type of specificity for base pairing potential has not been reported previously in any polymerase. These unique properties can explain how telomerase not only maintains fidelity of telomere synthesis but also has the capability for *de novo* telomere addition.

Results

Initiation of de novo telomere addition begins at the 3′ most templating nt 49

In *Tetrahymena*, as well as in other ciliates, the DNA sequences targeted for *de novo* telomere addition are A+T-rich non-telomeric sequences that most often end with a 3′ A or T residue (Yao *et al*., 1987; Yu and Blackburn, 1991). Recently, we showed, using *Tetrahymena* telomerase, that oligonucleotides consisting of these non-telomeric DNA target sequences can prime the addition of telomeric DNA efficiently *in vitro* (Wang and Blackburn, 1997). When *in vitro* telomere addition was assayed in the presence of [α-^{32}P]dGTP and unlabeled dTTP in a 'complete reaction', telomerase added multiple repeats of the *Tetrahymena* telomeric repeat unit GGGGTT. Substituting dTTP with ddTTP ('initiation reaction') causes termination of this *de novo* healing reaction after addition of the sequence dGGGGddT (hereafter referred to as G*/ddT reaction) (Wang and Blackburn, 1997). An example of the lack of a requirement for a base-paired 3′ end of the primer is shown in Figure 1, which compares utilization of two non-telomeric DNA substrates in *de novo* telomere addition. The primer N2(20) (where N indicates non-telomeric sequence, and the number in parenthesis the oligonucleotide length) is a natural *Tetrahymena* genomic sequence to which telomeric repeats are added *de novo in vivo* (Yao *et al*., 1987), and ends with -AAT-3′ (see Materials and methods and Figure 1 legend). The 3′ end T residue of primer N2(20) potentially could base-pair with any of the A residues on the telomerase RNA templating domain (3′-aaCCCCAAC-5′; Figure 1A). Primer N2(20)t/a was identical to N2(20) except that it ended in -AAA-3′ (Figure 1 legend), and therefore cannot base-pair with any template domain residue. However, both substrates were elongated efficiently and comparably by telomerase to produce similar +1 to +5 nt products in G*/ddT reactions (Figure 1B, lanes 2 and 4). The primer +1 to +4 nt products, corresponding to +G to +G_4, migrated identically to the primer +Gs markers (Figure 1B, compare lanes 1 and lane 2, and lanes 3 and 4). Significantly, the +5 nt product in both reactions reproducibly migrated faster than the primer plus G_5 marker, and from previous work was identified as the primer +G_4ddT product (Figure 1B, compare mobility of +G_5 marker in lanes 1 and 3 with product indicated by the arrowhead in lanes 2 and 4; Wang and Blackburn, 1997). As described previously, a primer-characteristic profile of +G to +G_4 products, in addition to +G_4ddT, results from non-processive elongation (Wang and Blackburn, 1997). In reactions with N2(20)t/a, we also

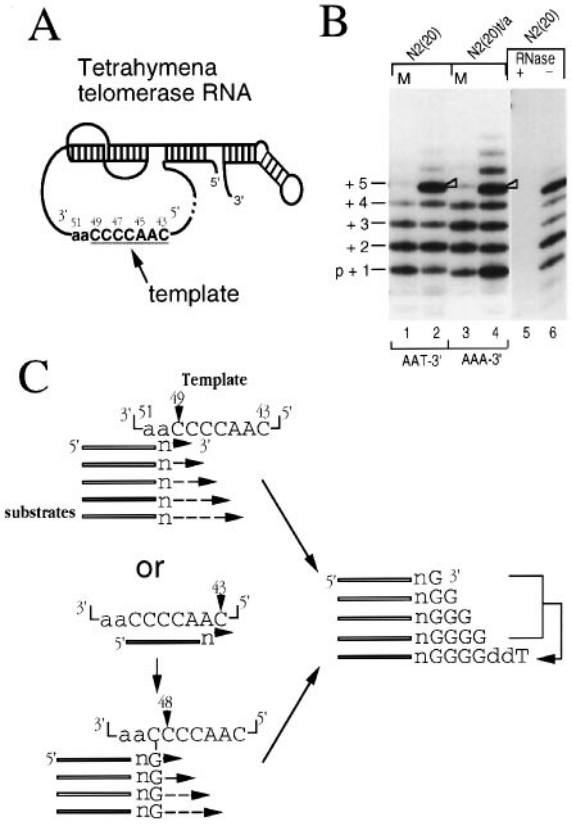

Fig. 1. Assay for *de novo* telomere addition onto non-telomeric DNA substrates. (**A**) Schematic secondary structure of *Tetrahymena* telomerase RNA (adopted from Gilley and Blackburn, 1996). Templating nucleotides are underlined. (**B**) Telomerase assays using non-telomeric primer substrates in [α-^{32}P]dGTP/ddTTP (G*/ddT) reactions. Primers were N2(20), 5′-GTTTA ATTTA AGAAA ATAAT-3′; and N2(20)t/a, 5′-GTTTA ATTTA AGAAA ATAAA-3′. The sequence at the 3′ end of each substrate is indicated below each lane. Reaction products were separated by denaturing polyacrylamide gel (15%) electrophoresis, and a scanned image of the autoradiogram of the gel is shown. Markers (M) were primer oligonucleotides 3′ labeled with [α-^{32}P]dGTP using terminal deoxynucleotidyl transferase (Life Technologies). (**C**) Two possible alignments of the 3′ end of a non-telomeric primer on the template that could initiate production of GGGGddT.

observed slightly higher amounts of +G_5ddT and +G_6ddT products (Figure 1B, band above arrowhead in lane 4) than in reactions with N2(20). This product appears to reflect product realignment or slippage during elongation. Quantitative analyses using N2(20)t/a and N2(20) in parallel competition assays (see Materials and methods) also showed that these two primer substrates were extended by telomerase with comparable efficiencies (data not shown). Therefore, a complete lack of potential of Watson–Crick base pairing to the template does not prevent *de novo* telomere addition by telomerase. We also examined the ability of telomerase to extend various non-telomeric primers, including those used in previous work (Wang and Blackburn, 1997). Our results consistently showed that, although the extension efficiency is affected by the 5′ sequence of the test primer, non-telomeric primers ending with mismatched A or C residues can be extended by telomerase, initiating with the sequence GGGGddT (data not shown).

We first determined which position on the template

Table I. Elongation of primer substrates with varying potential to base-pair with template nt 49–46

Primer substrates	Designed primer 3′ end on template		ddN termination product[c]	Inferred primer 3′ end on template	
	(RNA template) 3′-aaCCCCAAC-5′ (50–43)			3-aaCCCCAAC-5′ (50–43)	
N2(20)g	5′-gtttaatttaagaaaataatg[a,b]	49	GGGddT	-tg	49
N2(20)a	5′-gtttaatttaagaaaataata	49	GGGGddT	-ta	50
N2(20)gg	5′-gtttaatttaagaaaataatgg	48	GGddT	-tgg	48
N2(20)ga	5′-gtttaatttaagaaaataatga	48	GGGGddT	-tga	50
N2(20)cg	5′-gtttaatttaagaaaataatcg	48	GGGddT	-tcg	49
N2(20)ggg	5′-gtttaatttaagaaaataatggg	47	GddT	-tggg	47
N2(20)gga	5′-gtttaatttaagaaaataatgga	47	GGGGddT	-tggga	50
N2(20)gag	5′-gtttaatttaagaaaataatgag	47	GGGddT	-tgag	49
N2(20)agg	5′-gtttaatttaagaaaataatagg	47	GGddT	-tagg	48
N2(20)gggg	5′-gtttaatttaagaaaataatgggg[d]	46	TTddG	-tgggg	46
N2(20)ggga	5′-gtttaatttaagaaaataatggga	46	GGGGddT	-tggga	50
N2(20)ggag	5′-gtttaatttaagaaaataatggag	46	GGGddT	-tggag	49
N2(20)gagg	5′-gtttaatttaagaaaataatgagg	46	GGddT	-tgagg	48
N2(20)aggg	5′-gtttaatttaagaaaataatagggg	46	GddT	-tagggg	47

[a]All primer substrates have a N2(20) 5′ backbone sequence. The underlined base indicates the 3′ most mismatched residue in each substrate.
[b]Dotted lines indicate the positional base pairing.
[c]ddN represents dideoxynucleotide ddGTP or ddTTP.
[d]Each of this set of substrates was assayed in reaction with both [^{32}P]dTTP/ddGTP and [^{32}P]dGTP/ddTTP. For the two reactions for each substrate, only the one that had efficient substrate elongation is presented. All other substrates were assayed in reaction with [^{32}P]dGTP/ddTTP.

RNA is copied into the first nucleotide added *de novo*. Synthesis of the first added sequence, GGGGT, potentially could initiate at nt 49 and/or nt 43 on the template (schematic in Figure 1C). Initiation at template nt 49 would involve copying nt 49–45 into GGGGddT, while initiation nt 43C would most probably require the 3′ end of the +G product to translocate to template nt 49 and copy nt 48–45. To distinguish between these possibilities, telomerase reactions were performed using template mutant telomerases: mutant enzyme 43A, in which the original 43C was replaced with an A residue, and mutant enzyme 49G, in which the original 49C was replaced by a G residue (Gilley *et al.*, 1995; Gilley and Blackburn, 1996). If initiation occurred at position 43, wild-type and 43A mutant enzymes were expected to produce significantly different product profiles in the G*/ddT reaction. Conversely, initiation at position 49 predicted that the 49G enzyme would first add a C residue to a non-telomeric primer, by copying the mutant 49G position, whereas the products made by the 43A enzyme would be the same as wild-type enzyme products in the G*/ddT reaction.

We tested these predictions using the substrate N2(30) (-AAT-3′; Materials and methods), which is the same as N2(20) but contains an additional 10 nt of the natural sequence 5′ to the N2(20) sequence, and produces a similar product profile (Wang and Blackburn, 1997). With the 43A mutant telomerase, in G*/ddT reactions the profiles and ratios of the +1 to +5 nt products were similar to those produced by wild-type enzyme (Figure 2; cf. lanes 1 and 2). As ddT would block any product initiated at position 43 by the 43A enzyme, we also performed reactions containing *dGTP and TTP; again, the levels and profiles of the short products were similar to those in the G*/ddT reactions (data not shown). In analogous experiments performed using primer N2(30)t/a (-AAA-3′; see Materials and methods), the 43A enzyme

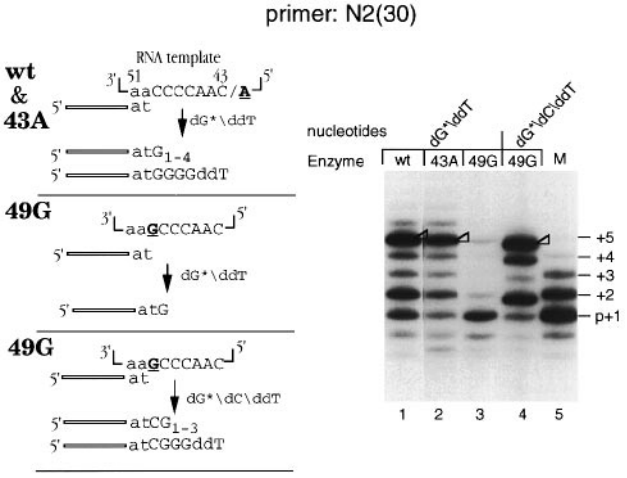

Fig. 2. Initiation of *de novo* telomere addition using telomerases containing template sequence mutations. Telomerase reactions were carried out with primer N2(30) (5′-TAAAT AGTTT GTTTA ATTTA AGAAA ATAAT-3′). Schematic: inferred alignments of the primer 3′ end based on the reaction products observed. Wild-type, mutant telomerases and nucleotides used in each reaction are indicated above the gel lanes. Markers (M) are as in Figure 1. A lighter exposure of gel lane 1 (wild-type enzyme reaction) is shown.

extension products in G*/ddT reactions again closely resembled those made by wild-type enzyme (data not shown). These results are consistent with the interpretation that initiation does not involve copying position 43 (Figure 2, see schematic). In a direct test for copying position 49, we compared the 49G enzyme with G*/ddT reactions and G*/ddT reactions supplemented with dCTP. In G*/ddT reactions, the only significant extension product of N2(30) was primer +G (Figure 2, lane 3). This apparently non-processive addition of a single G could have been templated by nt 48–46C or 43C, as this reaction was RNase sensitive (data not shown). Therefore, in the

absence of dCTP, the nucleoside triphosphate specifically required for copying template nt 49G, 49G enzyme failed to extend such a primer, or the +G product, productively into longer products. Including dCTP in the G*/ddT reaction restored extension of N2(30) by the 49G enzyme to a product profile similar to that of wild-type enzyme (Figure 2, compare lanes 1 and 4). Significantly, these labeled 49G enzyme products, from +2 nt onward, all consistently migrated faster than the primer $+G_{1–5}$ markers (Figure 2, compare lanes 4 and 5). This migration difference is indicative of incorporation of an initial dC residue into these products as described previously (Gilley and Blackburn, 1996), and hence of addition of the initial sequence CGGGddT. From these results, we conclude that *de novo* telomere addition begins primarily at template nt 49.

Incorporation of [^{32}P]dG residues in the initiation reaction by wild-type activity was RNase sensitive, as expected for a telomerase-mediated reaction (Figure 1B, lane 5). However, this did not eliminate the possibility that such G addition may take place by a non-templated mechanism. To test this possibility directly, we used a different mutant telomerase, which contained no rC residues in its templating domain. In this mutant telomerase, the seven nucleotide template sequence (3′-CCCCAAC-5′; telomerase RNA positions 49–43) was replaced with the sequence 3′-UAUAUAU-5′, with the rest of the RNA having the wild-type sequence. This ‘C-free template’ enzyme was catalytically active *in vitro* in reactions containing [^{32}P]TTP and dATP, with several telomeric and non-telomeric primer substrates ending with a 3′ T or A. However, it failed to add any non-templated [^{32}P]G residues (H.Wang and E.H.Blackburn, unpublished results). Taken together, the available evidence indicates that addition of G residues to non-telomeric primers by telomerase involves copying from the telomerase RNA template, initiating at position 49.

The requirement for potential pairing between primer 3′ end and template increases as catalysis moves along the template

Extension of non-telomeric primers from a single template position, nt 49, implies that during the initiation reaction the substrate’s mismatched 3′ end aligns opposite nt 50. Conceivably, this could be due to tolerance of a mismatch opposite nt 50, and/or a negative effect of a mismatch at other positions on the template. To examine these possibilities, we analyzed sets of substrate oligonucleotides with a non-telomeric 5′ region but varying potentials for base pairing between their 3′ nucleotides and template RNA nucleotides.

Figure 3 shows examples of these experiments. Primer N2(20)gg elicited addition of +G, $+G_2$ and $+G_2$ddT in the G*/ddT reaction (Figure 3A, lane 2; open arrowhead indicates product $+G_2$ddT). These were the products expected from alignment of the primer 3′ end at nt 48, via base pairing of its -tgg 3′ end with nt 50–48, and copying template nt 47–45. Such a $+G_2$ddT product is predicted, and was observed, to migrate faster than primer $+G_3$ marker (Figure 3A, cf. lanes 1 and 2). In contrast, with primer N2(20)ga, which differed from N2(20)gg only at its 3′ residue (a template-mismatched A residue; see Figure 3A), no identifiable +GGddT product was detected. This was confirmed by careful examination of varying autoradiographic exposures of the sequencing gels used to fractionate the products, under conditions in which the $+G_3$ marker and +GGddT product showed a distinct mobility difference (Figure 3A, compare lanes 3 and 4; data not shown). Instead, N2(20)ga primed synthesis of +G, $+G_2$, $+G_3$ products, and lower amounts of $+G_4$ and $+G_4$ddT products ($+G_4$ddT is indicated by the arrowhead in Figure 3A, lane 4). This pattern of products indicated that elongation from this primer initiated by copying position 49, implying that the mismatched A residue was positioned opposite nt 50.

Primer N2(20)gagg, differing from primer N2(20)gggg by a single base substitution, has a 3′ end which can be aligned at nt 46 of the template, maximizing potential base pairing between the primer 3′ end region and the templating domain of the RNA. Such an alignment would position a mismatched A residue at template nt 48 (schematic in Figure 3B). However, this primer was elongated efficiently in G*/ddT reactions to form the termination product $+G_2$ddT (Figure 3B, lane 2; see also below), suggesting that the mismatched A residue was uniquely positioned at RNA nt 50 and the 3′ end was aligned at nt 48. As exemplified by this experiment, we systematically examined the tolerance for a mismatched base opposite this and other positions of the template. In reactions containing [α-^{32}P]TTP and ddGTP (T*/ddG reactions) with primer N2(20)gggg, whose 3′ region can completely base-pair with template nt 46–50 (schematic in Figure 4A), +T, +TT and +TTddG products were synthesized as expected (Figure 4A, lane 1). These are the predicted products from alignment of its 3′ end at nt 46. We noted that despite the perfect match of the 3′ region of this primer with the template, the most prominent product was primer +T, rather than +TT and +TTddG. This low processivity is consistent with the lack of a strong interaction between the anchor site of telomerase and the 5′ non-telomeric sequence of this primer as described previously, and is still observed, although partially alleviated, even at non-limiting TTP concentrations (Lee and Blackburn, 1993). However, primer N2(20)gggg was elongated very inefficiently in the G*/ddT reaction (Table I and data not shown). The opposite result was obtained with the set of N2(20)gggg-derived substrates which each contained a single mismatch targeted to positions 46–49 (Table I). As described above for N2(20)gagg, each of these mismatch-containing substrates was extended efficiently by positioning its 3′-most mismatched residue at nt 50 of the RNA, as deduced from the products made in G*/ddT reactions (Table I). In contrast, as revealed by T*/ddG reactions, efficient elongation of these substrates based on alignment of their 3′ ends at template nt 46 did not occur (Figure 4A, lanes 2–5). We noted that there was a detectable elongation from position 46 with a 3′ mismatched A residue at position 46 [primer N2 (20)ggga]. This suggested some partitioning between two alignments: one with the 3′ end at position 50, and a minor fraction with the 3′ A at position 46. However, with a mismatched C instead of an A, the T*/ddG reaction signal was even lower (data not shown). In contrast, introducing a mismatch at position 50 had only a modest effect on copying of position 45 (Figure 4A, lanes 6 and 7). Hence, the only alignment of the 3′ end of each mismatched primer that

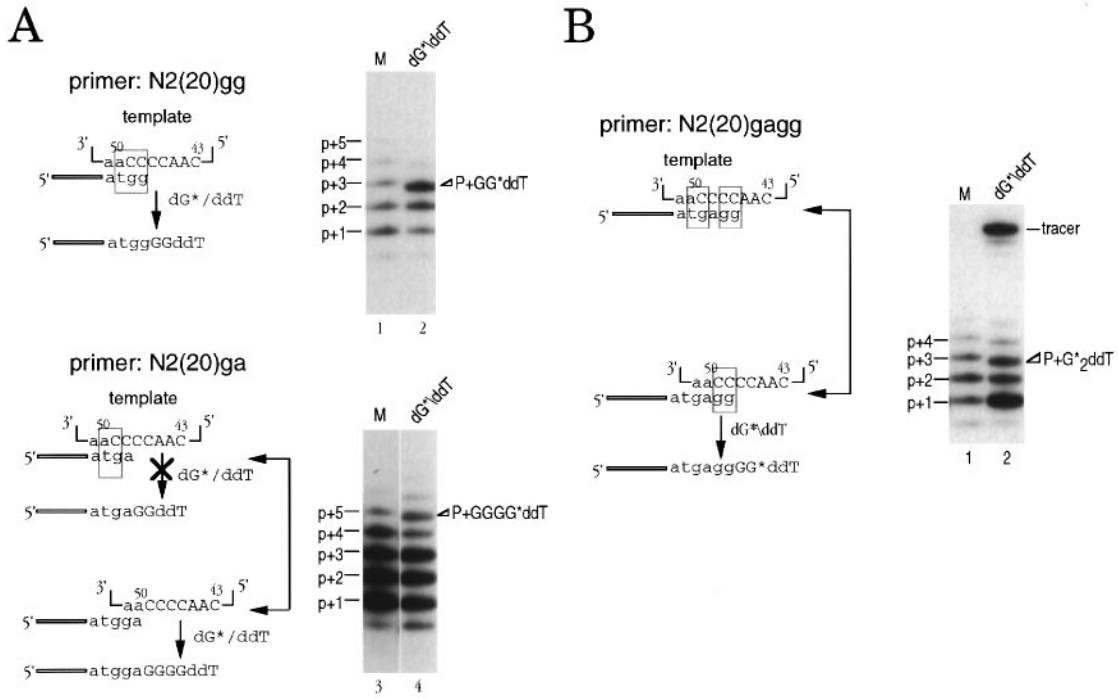

Fig. 3. Elongation of substrates with the potential for partial base pairing with template nucleotides 49–46. Schematics on the left of each panel indicate the putative alignment of the primer 3′ end on the template based on maximal possible base pairing (top) or the alignment inferred from actual products (bottom). Matched template–primer positions are boxed. Markers (M) as in Figure 1. (**A**) Telomerase assays of primers N2(20)gg and N2(20)ga in the G*/ddT reaction [lighter exposures showed no apparent GGddT product in the reaction with N2(20)ga; data not shown]. (**B**) Telomerase assays of primer N2(20)gagg in the G*/ddT reaction as indicated. Tracer indicates a ^{32}P-labeled DNA oligonucleotide (37mer), included in each reaction mix after termination of the assay, used for monitoring the recovery of the reaction products.

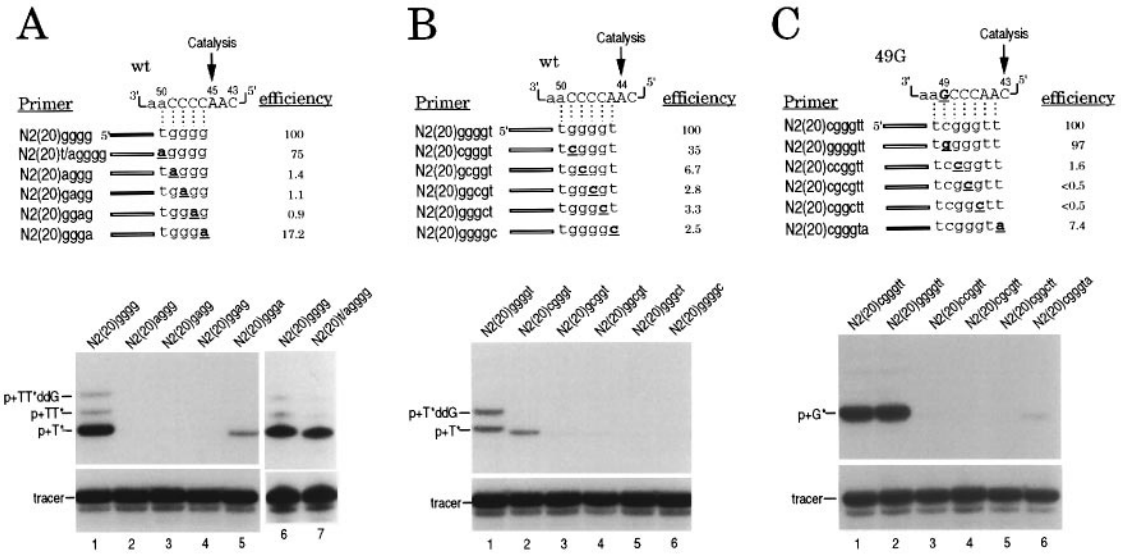

Fig. 4. Requirement for pairing between template and primer when copying template nt 45, 44 and 43. Schematics above each panel indicate the predicted alignment of the primer 3′ end based on maximal possible base pairing potential with wild-type or 49G telomerase RNA template. In each set of primers, the efficiency of elongation for each primer is quantified and normalized against the 100% value for the perfectly matched primer [N2(20)gggg, N2(20)ggggt and N2(20)cgggtt respectively in (A), (B) and (C)]. The mismatched nucleotide in primers and the mutated template nt 49G are shown in bold and underlined. The tracer in each panel is as in Figure 3B. (**A**) and (**B**) T*/ddG reactions with wild-type enzyme. Assays for (A) and (B) were carried out in a single experiment with the same autoradiographic exposure, except for lanes 6 and 7 in (A) which were a separate experiment. (**C**) G*/ddT reactions with 49G mutant enzyme.

allowed efficient and productive elongation was that which positioned the mismatch opposite nt 50 [e.g. see schematic for N2(20)gagg, Figure 3B]. Notably, other possible alignments of the 3′-gg of N2(20)gagg (at 46 and 47, or at 47 and 48) would also have allowed these two 3′ bases to pair with the template, but these alignments were not utilized by telomerase. Therefore, in addition, this result suggested that a mismatch opposite template nt 49–46 is not tolerated.

In comparable sets of experiments, similar results were found for sets of substrates derived from N2(20)g, N2(20)gg and N2(20)ggg (Table I), each containing single

G→A substitutions. If primer–template domain pairing were maximized, the 3′ ends of these substrates would have aligned at template nt 49, 48 and 47 respectively (Table I). However, in *G/ddT reactions, each of these G→A-substituted substrates primed synthesis of the products predicted if each primer's 3′-most mismatched residue aligned opposite nt 50, followed by copying of template nucleotides and terminating at position 45.

Together, the results shown in Figures 3 and 4A and summarized in Table I showed that even though the mismatch-containing primers tested had more than one possible alignment by base pairing on the template, efficient elongation by telomerase only took place from an alignment that does not result in even a single mismatch positioned opposite nt 46–49 of the template. Therefore, we conclude that such mismatches prevent polymerization. These results suggest that, for this group of mismatch-containing primers, two criteria determine its mode of elongation: first, that the 3′ most mismatch residue be no closer to the templating residues than nt 50; and, only secondarily, that the degree of template–primer base pairing be maximized. Thus, as long as the condition of positioning the mismatch to nt 50 is met, as little as one potential base pair can dictate the position of alignment on the template.

Experiments were next performed to define the specific requirements for base pairing with the template as telomerase copies along the template. We compared two sets of mismatched primer substrates derived from the matched primers N2(20)ggggt and N2(20)ggggtt, whose 3′ ends can align at template nt 45 and 44 respectively. For each set of primers, a mismatch residue was placed at varying distances from the 3′ end of the primer (schematic in Figure 4B and C). The 3′ nucleotide was designated the –1 position on the primer, and the neighboring more internal positions of the primer –2, –3, etc. Primer N2(20)ggggt, which has a perfect match to template nt 45–50, elicited relatively efficient addition of both +T and +TddG in T*/ddG reactions, through copying of positions 44 and 43 (lane 1, Figure 4B). However, the efficiency of elongation of mismatched substrates derived from N2(20)ggggt, N2(20)ggggc and N2(20)gggga was ~2% of that of the perfectly matched substrate N2(20)ggggt (Figure 4B, lane 6 and data not shown). These results were also confirmed by parallel competition assays (see Materials and methods; data not shown). Locating the mismatched dC residue progressively further away from the 3′ end of the primer, at positions –2, –3 and –4 [primers N2(20)gggct, N2(20)ggcgt and N2(20)gcggt], reduced extension levels to 2–7% of that of the fully matched primer N2(20)ggggt (lanes 3–6 and table in Figure 4B). Therefore, single mismatches introduced at positions –1 to –4 on the primer blocked synthesis initiated by copying template position 44. These results suggested that copying position 44 requires at least the four contiguous 3′ end nucleotides of the primer to have the potential to pair with template positions 45–48. Notably, moving the internal mismatch to position –5 [primer N2(20)cgggt] partially restored synthesis of the first (+T*) product at position 44, but now synthesis of the +2 product (T*ddG) by copying position 43 was barely detectable (Figure 4B, lane 2). This result suggested that the second polymerization event, at position 43, was significantly more sensitive to the mismatch at position 49 than the initial polymerization event at position 44.

To analyze the effects of mismatches in the set of primers whose 3′ end potentially could align to template nt 44 [N(20)ggggtt and derivatives], we needed to be able to distinguish between products formed from copying position 43 versus other positions. Therefore, we used G*/ddT reactions with the mutant 49G enzyme, and T*/ddG reactions with the mutant 43A enzyme. In each case, a prominent labeled +1 product (+G* with 49G, and +T* with 43A) would be synthesized if position 43 were copied. Mismatches located at position –1, –3 –4 and –5 on the primer [N2(20)cgggta, N2(20)cggctt, N2(20)cgcgtt and N2(20)ccggtt respectively] all severely suppressed catalysis compared with the perfectly matched control primer N2(20)cgggtt (49G enzyme; Figure 4C, lanes 3–6). In contrast, a mismatch at position –6 [primer N2(20)ggggtt with 49G enzyme] restored polymerization to 97% of the level of the control primer N2(20)cgggtt (Figure 4C, lanes 1 and 2). Similar results were obtained with the 43A enzyme in T*/ddG reactions, using a set of primers with either a complete match to template nt 50–44, or single mismatches targeted to nt 49–44. Specifically, while again a mismatch at position –1 [N2(20)ggggta] severely suppressed *dT addition copying template position 43, extension of a primer with an internal mismatch at position –6 [N2(20)cgggtt] was 76% that of the control primer N2(20)ggggtt (data not shown). Thus, pairing with template nt 49 was not required when the initial polymerization (+1 product formation) involves copying position 43. Therefore, we conclude that such polymerization at position 43 requires five, but not six, base pairs of the primer 3′ end to be able to form a perfect duplex with template nt 44–48.

Taken together, these results and those presented in Figure 3 and Table I revealed that as polymerization moves along the template from position 49 to position 43, the required number of potential contiguous base pairs between the 3′ end of the primer and the templating domain increases from no base pairs, for polymerization at position 49, up to five pairs for polymerization at position 43.

Discussion

For many DNA polymerases, productive elongation of a primer substrate requires a minimal length of the 3′ end of the primer to be in a duplex conformation with the template (Nevinsky *et al.*, 1990). Here we report that with *Tetrahymena* telomerase, unlike with other DNA polymerases or reverse transcriptases, the negative impact of a mismatch on polymerization is not determined by its distance from the 3′ end of the primer, but rather by which template position is in the catalytic site of the enzyme. As the catalytic site of telomerase successively copies template nt 49, 48, 47, 46, 45, 44 and 43, respectively, it requires minimally zero, one, two, three, four, four and five contiguous potential base pairs between the primer 3′ end and the template (Figure 5A). A crucial aspect of telomerase, which very probably underlies these unusual requirements, is that the template region, which is a small part of its longer, built-in RNA, is constrained by being held in the RNP. Since the template is an intrinsic

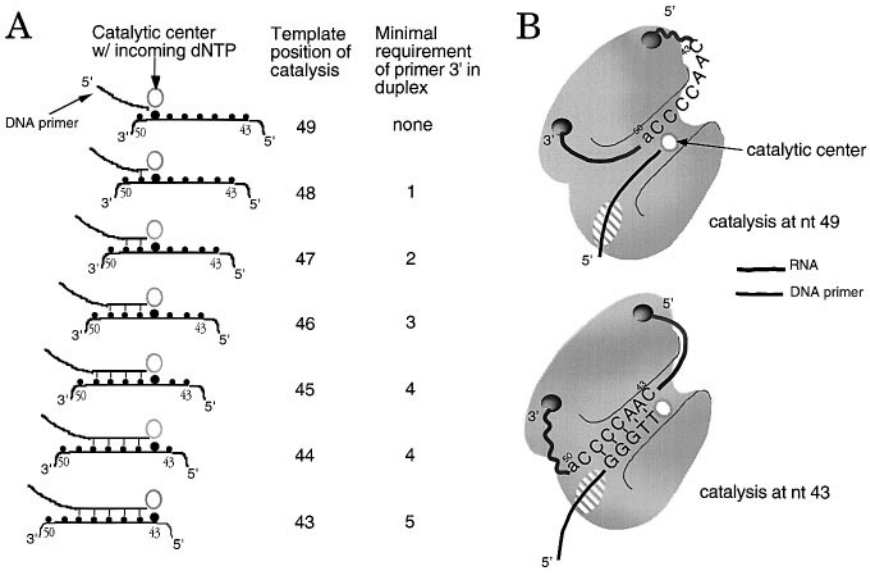

Fig. 5. Position-specific template–DNA pairing requirements for telomerase function. (**A**) The minimal requirement for template–DNA substrate pairing for catalysis by telomerase at specific template positions is shown. (**B**) A spatial constriction model for movement of the telomerase RNA template through the catalytic site. The constraint on template movement within the telomerase RNA is depicted as the binding sites (dark spots) on both ends of the RNA region encompassing the template region (thick dark line). Hatched area: the anchor site of telomerase that can interact with the single-stranded primer substrate [thin dark line (Collins and Greider, 1993; Lee *et al.*, 1993; Wang and Blackburn, 1997)].

part of the telomerase RNP, the spatial relationship between the polymerization site and the built-in telomerase RNA must inherently change as each RNA template position moves into the active site. Consistent with this view, another active site property, the efficiency of utilization of nucleoside analog triphosphate inhibitors, is also highly dependent on the template position being copied (Strahl and Blackburn, 1994).

For other DNA polymerases, besides contributing to the stability of the polymerase–template–primer ternary complex (Nevinsky *et al.*, 1990), duplex formation between the primer 3′ end and the template is an important prerequisite for an induced conformational change occurring prior to the catalytic step of polymerization (Johnson, 1993). This conformational change may help in selecting the correct incoming nucleotide substrate (Petruska *et al.*, 1988). It also is thought to transform the active site from an 'open' inactive form to a 'closed' active form immediately before the catalytic step (Johnson, 1993). These built-in mechanisms contribute to replication fidelity in a variety of DNA polymerases. Therefore, we suggest that with telomerase, the primer 3′ end can become stabilized on a particular template position, thereby allowing that position to be copied, only if such a pre-catalytic conformational change of the telomerase RNP can occur. We propose that a mismatch between the primer–template duplex can destabilize or prevent the required conformational change(s) in a manner that depends specifically on which template position is in the catalytic site. In this model, alignment of the mismatch to nt 50 of the RNA distorts the telomerase active site the least compared with mismatches at other template positions, and thus is the least disruptive of productive elongation. Since catalysis cannot take place until a kinetically favorable alignment occurs, the *de novo* telomere addition pathway, beginning by copying position 49, occurs by default.

A possible model for the structural basis for such template position-specific effects is shown schematically in Figure 5B. When position 49 is in the catalytic active site, all the template RNA residues are on the downstream side of the active site. We propose that as polymerization proceeds along the template, a template–product duplex builds up on the upstream side of the catalytic site. As polymerization progresses up to position 44, more RNA, as well as the newly synthesized DNA, has to be accommodated on the upstream side of the catalytic site. Thus, closing into the active conformation is predicted to become progressively more sterically restricted by this increasing bulk of RNA plus DNA, and hence less tolerant of mismatches. However, when the catalytic site reaches position 43, position 49 no longer has to be paired with the DNA. Hence, we suggest that at this point, position 49 has moved out of the sterically restricted region and the DNA at this position can interact with the anchor site (Figure 5B, hatched area).

It is notable that the drastic change in mismatch tolerance by the telomerase active site demonstrated here coincides with the previously defined 3′ boundary of the template (Gilley and Blackburn, 1996). Therefore, both the unavailability of position 50 to the catalytic site and its mismatch tolerance might have the same underlying basis, in that both may occur because a close grip of the telomerase active site on the RNA bases outside the 3′ boundary of the RNA template is prevented by steric hindrance. Alternatively, the tolerance for a mismatch at position 50 might be mediated by a special conformation when the active site is at this position, possibly facilitated by protein–RNA interactions.

The results reported here have relevance for understanding the underlying mechanism of *de novo* telomere addition onto non-telomeric DNA primer substrates with limited, or no, potential for base pairing with the telomerase RNA template. Our findings with *Tetrahymena* telomerase may

extend to other telomerases. In *Paramecium* and *Plasmodium*, many of the healing sites analyzed have no telomeric sequences. Most frequently, telomere addition initiated with addition of three dG residues, consistent with copying the 3′ most portion of the template (Forney and Blackburn, 1988; Scherf and Mattei, 1992; Scherf *et al.*, 1992). Therefore, telomerase from those species might closely resemble that of *Tetrahymena* by lacking a requirement for base pairing at a unique position, which allows non-telomeric primers to be extended. In contrast, yeast and human telomerase healing events *in vivo* appear to have extended primer substrates whose 3′ ends can form three or more base pairs at any of a variety of positions on the template (Wilkie *et al.*, 1990; Kramer and Haber, 1993; Lamb *et al.*, 1993; Flint *et al.*, 1994). Hence, mismatch intolerance over at least 3 or 4 template bases may be a functionally conserved feature of telomerases.

Materials and methods

Cells

Tetrahymena strains, conjugation and maintenance were as described (Wang and Blackburn, 1997). Construction of telomerase RNA genes with 43A and 49G, the C-free template mutant and transformation of *Tetrahymena* were as described previously (Gilley and Blackburn, 1996).

Partial purification of telomerase

S100 cell extracts of 9–11 h mated wild-type cells, or vegetative cells transformed by each mutant telomerase RNA construct and starved for 12–24 h, were prepared as described (Greider and Blackburn, 1987). Chromatographic purification of telomerase from S100 via DEAE–agarose and heparin–agarose were as described (Wang and Blackburn, 1997).

Telomerase assays

Telomerase reactions were carried out in a 10 or 20 μl reaction mix containing 50 mM Tris–HCl, pH 8.3, 1.25 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 100 μM ddTTP (or ddGTP), 1.25 μM [α-^{32}P]dGTP or [α-^{32}P]dTTP (Dupont NEN, Boston, MA, 800 mCi/mmol) and DNA primers (200 μM). Reactions were initiated by addition of wild-type or mutant enzyme fractions. Reactions were incubated at 30°C for 10 min, under which conditions the telomerase reaction is in the linear range (Wang and Blackburn, 1997; and unpublished). All reactions were terminated by addition of 80 μl of TES (50 mM Tris–HCl, pH 8.0, 20 mM EDTA, 0.2% SDS) and phenol–chloroform extraction. For the experiments shown in Figures 3 and 4, a 5′-labeled tracer DNA (37mer) was added to each reaction mix after adding TES at the end of the reaction. Reaction products were precipitated by EtOH and were separated on 15% denaturing polyacrylamide gels followed by X-ray autoradiography. The scanned images of the X-ray autoradiograms (scanned at 300 d.p.i. resolution) are presented in Figures 1–4 using a phaser 440 printer (Tektronix Inc., Wilsonville, OR). Quantitations of products on these gels were made using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). All experiments were repeated to ensure reproducibility. RNase and RNasin pre-treatment of telomerase in Figure 1 was as described (Cohn and Blackburn, 1995). All synthetic DNA oligonucleotides were purified by denaturing gel electrophoresis. The concentration of purified oligonucleotides was calculated based on 1 OD_{260} = 20 μg/ml of DNA and the molecular weight of the individual oligonucleotide. Primers were: N2(20), 5′-GTTTA ATTTA AGAAA ATAAT-3′; N2(20)t/a, 5′-GTTTA ATTTA AGAAA ATAAA-3′; N2(30), 5′-TAAAT AGTTT GTTTA ATTTA AGAAA ATAAT-3′; N2(30)t/a, 5′-TAAAT AGTTT GTTTA ATTTA AGAAA ATAAA-3′. All other primer substrates are derivatives of N2(20) and are shown in the figures or figure legends.

Parallel competition reactions

Each primer in a set of two or more substrates (‘test primers’), all having the same length but differing by a single nucleotide, at a fixed concentration was mixed with a common second primer, the ‘competing primer’ and assayed. The competing primer was longer (by 6–7 extra nucleotides on its 5′ side) but shared the same 3′ end sequence with the ‘matched’ test primer. All assays were carried out in the linear range for product synthesis: 10 min at 37°C (Wang and Blackburn, 1997). The concentration of each primer used was first determined empirically, such that the levels of products from the matched test primer were comparable with those from the competing primer. Products from each substrate were quantified (rate of extension) as either total products up to the first dideoxy terminated product, or the first dideoxy terminated product alone. Both ways of quantitation gave very similar results. The rate for the longer, 3′ matched competing primer in each reaction was divided by that for the test primer to generate a substrate specificity for the test primer (Fersht, 1984). Primer specificities for the matched and mismatched test primer substrates were compared.

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