Utilization of ribonucleotides and RNA primers by *Tetrahymena* telomerase

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Telomerase is a ribonucleoprotein (RNP) DNA polymerase involved in telomere synthesis. A short sequence within the telomerase RNA component provides a template for *de novo* addition of the G-rich strand of a telomeric simple sequence repeat onto chromosome termini. *In vitro*, telomerase can elongate single-stranded DNA primers processively: one primer can be extended by multiple rounds of template copying before product dissociation. Telomerase will incorporate dNTPs or ddNTPs and will elongate any G-rich, single-stranded primer DNA. In this report, we show that *Tetrahymena* telomerase was able to incorporate a ribonucleotide, rGTP, into product polynucleotide. Synthesis of the product [d(TT)r(GGG)]_n was processive, suggesting that the chimeric product remained associated with the enzyme both at the active site and at a second, previously characterized, template-independent product binding site. As predicted by this finding, RNA-containing oligonucleotides served as primers for elongation. More than 3 nt of RNA at a primer 3' end decreased the quantity of product synthesis but increased the affinity of the primer for telomerase. Thus, RNA-containing primers were effective as competitive inhibitors of DNA primer elongation by telomerase. These results support the possible evolutionary origin of telomerase as an RNA-dependent RNA polymerase.

Keywords: polymerase/rGTP/RNA/telomerase

Introduction

Replication of linear chromosomes by DNA-dependent DNA polymerases is predicted to result in under-replication of the lagging strand (Watson, 1972). The 5' end of the lagging strand, after degradation of the most terminal RNA primer, can not be completed without an additional 5' Okazaki fragment. A solution to this end-replication problem was suggested by an experiment in yeast: linear plasmids with terminal *Tetrahymena* telomeric repeats were stabilized by the *de novo* addition of yeast telomeric simple sequence repeats (Shampay *et al.*, 1984). A similar *de novo* addition of telomeric repeats onto substrate primers *in vitro* was identified in extracts from the ciliate *Tetrahymena* (Greider and Blackburn, 1985).

This *de novo* telomeric repeat synthesis activity, termed

telomerase, has subsequently been demonstrated in extracts from a variety of organisms and cells including other ciliates, human, mouse and *Xenopus* (Zahler and Prescott, 1988; Morin, 1989; Shippen-Lentz and Blackburn, 1989; Prowse *et al.*, 1993; Mantell and Greider, 1994). The level of telomerase activity in cells or tissues is correlated with the maintenance or shortening of telomere length with cell proliferation (for a review, see Greider, 1994). High levels of telomerase activity have been described in cancer cells and low levels in normal blood cells, although most human somatic cell types are telomerase-negative (Counter *et al.*, 1994, 1995; Kim *et al.*, 1994). If telomerase activation aids in the continuous growth of transformed mammalian cells, telomerase inhibitors would be candidates for anti-cancer therapy (Harley *et al.*, 1994).

Telomerases contain essential RNA and protein components. RNA components have been cloned from numerous ciliates and from yeast (Greider and Blackburn, 1989; Shippen-Lentz and Blackburn, 1990; Romero and Blackburn, 1991; Lingner *et al.*, 1994; Melek *et al.*, 1994; Singer and Gottschling, 1994; McCormick-Graham and Romero, 1995). All telomerase RNAs contain a short internal sequence which serves as a template for synthesis of the G-rich strand of a telomeric repeat. Phylogenetic analysis of ciliate telomerase RNAs revealed that these RNAs share only a low level of primary sequence homology. However, some conservation of predicted secondary structure elements was observed (Romero and Blackburn, 1991; Lingner *et al.*, 1994; McCormick-Graham and Romero, 1995). Recently, the protein components of *Tetrahymena* telomerase have been identified and cloned (Collins *et al.*, 1995). The *Tetrahymena* telomerase RNP contains a 95 kDa protein (p95), an 80 kDa protein (p80) and the 159-nt telomerase RNA. The two protein components have unique nucleic acid binding properties: p95 binds specifically to telomeric primer DNA, whereas p80 binds most specifically to the telomerase RNA.

In vitro, *Tetrahymena* telomerase elongates G-rich, single-stranded DNA primers. With an 18-nt primer of three *Tetrahymena* telomeric simple sequence repeats, for example d(G₄T₂)₃, product DNA can be synthesized processively: the template can be copied multiple times during elongation of a single primer (Greider, 1991). Shorter primers are elongated less processively, with only one repeat of product DNA added to each primer before product dissociation (Collins and Greider, 1993; Lee and Blackburn, 1993). *In vivo*, the *de novo* synthesis of *Tetrahymena* macronuclear telomeres occurs in a relatively distributive manner (Yu *et al.*, 1990; Yu and Blackburn, 1991), similar to the elongation of short primers *in vitro*. *Tetrahymena* telomerase also catalyzes a nucleolytic cleavage reaction, removing 3' residues from primer or product DNA (Collins and Greider, 1993). The properties of the nucleolytic cleavage reaction catalyzed by telomer-

ase are similar to those of the nucleolytic cleavage reactions catalyzed by DNA-dependent RNA polymerases (for a review, see Kassavetis and Geiduschek, 1993). Cleavage by both enzymes is enhanced when product synthesis is stalled, for example by lack of a nucleotide or of template for additional synthesis. This cleavage reaction is likely to be catalyzed by the same active site as polymerization, in contrast with the 3'-5' exonuclease activity of DNA-dependent DNA polymerases, yet is distinct from pyrophosphorolysis (for a review, see Kassavetis and Geiduschek, 1993).

The similarity of models for processive product elongation and nucleolytic cleavage by the RNA polymerases (for example, Borukhov *et al.*, 1993; Johnson and Chamberlin, 1994) and telomerase (Collins and Greider, 1993) led us to investigate whether telomerase might also share other properties of an RNA polymerase. We assayed the incorporation of ribonucleotides and the elongation of RNA primers by *Tetrahymena* telomerase. Telomerase was surprisingly efficient as an RNA polymerase. This may reflect unknown functions or properties of telomerase required *in vivo*. In addition, the similarity of telomerase mechanism and specificity to that of RNA polymerases may be the result of evolution. Our observations suggest that the telomerase enzyme may have been adapted from an RNA-dependent RNA polymerase into the machinery for stable maintenance of linear DNA chromosome ends.

Results

Deoxy- and ribo-nucleotide titrations in quantitative telomerase assays

To perform a quantitative study of telomerase nucleotide specificity, we first determined appropriate reaction conditions. It was essential to characterize the linear range of enzyme activity, so that any change in the number of reactive enzyme molecules would be reflected in a change in activity. Telomerase enzyme concentration was estimated by Northern analysis from the concentration of telomerase RNA in extracts relative to standards of T7-transcribed telomerase RNA of known concentration (Collins *et al.*, 1995). In general, after ~100-fold purification of telomerase from crude cytoplasmic extract, activity increased linearly with enzyme concentration in the range of ~0.02–0.1 ng telomerase RNA per μ l final reaction volume (data not shown). After additional purification, no change was observed in reaction linearity or concentration dependence for primers or nucleotides using telomerase purified ~100-fold relative to highly purified telomerase (Collins *et al.*, 1995; data not shown). Also, for quantitative assays, telomeric DNA primers of 12–18 nt were assayed at concentrations below saturation (~1 μ M) to allow any decrease in primer binding to be reflected in decreased activity.

Using these conditions, we assayed elongation of the primer dG₂(G₃T₂G)₃ using either a fixed concentration of [α -³²P]TTP and titrations of dGTP or rGTP (Figure 1), or a fixed concentration of [α -³²P]dGTP and titrations of TTP, dUTP or rUTP (Figure 2). Product was analyzed in two ways. Reactions were precipitated and product was separated by denaturing gel electrophoresis to examine elongation processivity (Figures 1A and 2A). Aliquots of reaction products were also bound to DE81 paper, washed,

and quantitated to determine total radiolabeled product (Figures 1B and 2B). In this manner, both the pattern of product elongation and the overall elongation efficiency could be determined.

In reactions with [³²P]TTP, a small amount of two DNA products was produced in the absence of a guanosine nucleotide (Figure 1A, arrowheads). Their synthesis resulted from telomerase-mediated cleavage of the primer 3' end residues TTG and re-addition of [³²P]TTP, creating radiolabeled products shorter by 1–2 nt than the original primer (Collins and Greider, 1993, and data not shown). In the presence of increasing dGTP concentrations, a 6-nt ladder of products was observed. This ladder results from the preferential dissociation of product (Lee and Blackburn, 1993) and/or pausing of synthesis (Greider, 1991) at the 5' end of the template RNA during processive elongation. Only 1 μ M dGTP was sufficient for the processive synthesis of telomeric repeats, although a 6-nt ladder of product DNA was observed at up to 100 μ M dGTP (Figure 1A). At 1 mM dGTP, product synthesis was decreased, and a 1-nt ladder replaced the typical 6-nt repeat (see Discussion). At 10 mM dGTP, synthesis of radiolabeled product DNA was inhibited almost completely. The inhibition of product synthesis with increasing dGTP was evident at concentrations as low as 100 μ M (Figure 1B). Quantitation by filter binding of repeated assays revealed that between 1–10 μ M dGTP was optimal. This measurement agrees with similar values previously obtained from a different assay (Lee and Blackburn, 1993).

In the presence of [³²P]TTP and rGTP, a low level of processive synthesis was generally detected at 10–100 μ M rGTP (Figure 1A). Synthesis of product was enhanced by increasing rGTP to 1–10 mM. However, even at an optimal rGTP concentration of 1–10 mM, less product was synthesized than in the presence of dGTP (Figure 1B). The rGTP used in these experiments should be free of dNTP contaminants (see Materials and methods). However, we performed several controls to ensure that the products observed resulted from incorporation of rGTP. Incorporation of rGTP was observed for multiple preparations of telomerase, including telomerase extensively purified to near homogeneity (Collins *et al.*, 1995; Figure 1C). Products from reactions of highly purified telomerase with [³²P]TTP and rGTP, with the exception of the products resulting from addition of only TTP to primer DNA, were sensitive to post-treatment of the reaction with guanosine-specific RNase T1 (Figure 1C; products from addition of TTP alone are indicated by arrowheads). In the reactions in Figure 1C and others not shown, DNA products were not sensitive to RNase T1 digestion. Thus, the product ladders synthesized in the presence of rGTP contained incorporated ribonucleotides.

In the presence of [³²P]dGTP with no other nucleotide, significant product synthesis onto the primer dG₂(G₃T₂G)₃ was observed (Figure 2A). However, this product DNA, which probably represents a ladder of single nucleotide dGTP addition (see Discussion), was of limited length and did not demonstrate the 6-nt repeat pattern characteristic of synthesis of telomeric repeats. In the presence of [³²P]dGTP and TTP, product DNA with a 6-nt ladder was observed at TTP concentrations between 1 μ M and 10 mM (Figure 2A). However, as in reactions with high dGTP concentrations, product synthesis was inhibited at 1–10 mM TTP

Fig. 1. Titration of rGTP or dGTP in telomerase reactions. The indicated concentrations of rGTP or dGTP were added to assays in the presence of 2.5 μ M [32 P]TTP. A 1 μ M concentration of dG₂(G₃T₂G)₃ primer was used. Reactions contained additional MgCl₂ to compensate for nucleotide titrations, as indicated in Materials and methods.

(A) Product was precipitated and analyzed by electrophoresis on a 10.5% acrylamide, 7 M urea gel. The exposure time of the rGTP half of the gel is approximately four times that of the dGTP half of the gel, to allow visual interpretation of both titrations. Products resulting from primer cleavage and addition of [32 P]TTP only are indicated by arrowheads. (B) Product from reactions at the indicated nucleotide concentrations was spotted on DE-81 paper, washed, and quantitated. Scale is in c.p.m. $\circ$, rGTP reactions; $\square$, dGTP reactions.

(C) Reactions with highly purified telomerase, [32 P]TTP, and the indicated concentrations of rGTP were stopped by the addition of EDTA. Product was either mock-treated (-) or treated (+) with RNase T1 before precipitation and electrophoresis on an 8% acrylamide, 7 M urea gel. All lanes are from the same autoradiographic exposure of the same gel; RNase T1 treatment reduced the background radioactivity of samples in the T1-treated gel lane. Products resulting from primer cleavage and addition of [32 P]TTP only are indicated by arrowheads. This preparation of telomerase was less active than that used in (A) and (B).

(Figure 2B). Quantitation by filter binding of repeated experiments indicated that 10–100 μ M TTP was optimal, consistent with the 10-fold higher nucleotide concentration requirement for dTTP reported previously (Lee and Blackburn, 1993). Substitution of dUTP for TTP produced virtually identical product DNAs (Figure 2A), in only slightly reduced amount (Figure 2B). In contrast, no alteration of the [32 P]dGTP alone pattern was observed with titration of rUTP (Figure 2A), except at high concentrations (1–10 mM) where inhibition of product synthesis was observed (Figure 2A and B).

Elongation of RNA and chimeric RNA–DNA primers

Telomerase binds to product at two sites: the active site and a second, template-independent site termed the anchor site (Collins and Greider, 1993). Product bound at the anchor site can remain associated with telomerase even when the active site releases the product 3' end to reposition it for another round of template copying. This

promotes the processive elongation of product DNA. Thus, the processive elongation of ribonucleotide-containing product (Figure 1A) suggested that the telomerase anchor site must have a substantial affinity for the r(G)d(T) polymer. We reasoned that if the anchor site had significant affinity for RNA, then primers composed entirely or partly of RNA might be elongated.

The elongation of RNA and RNA–DNA chimeric primers by telomerase was assayed over a range of primer concentrations and tested for sensitivity to pre-treatment of telomerase with micrococcal nuclease (MNase), which inhibits telomerase-mediated elongation by degrading the essential RNA component of the enzyme. Elongation of the primers r(GU₂G₃)₂, d(GT₂G₄)r(U₂G₃), r(G₃U₂G)₃ and d(G₃T₂G₄)r(U₂G₄U₂G) was observed and was sensitive to MNase pre-treatment of telomerase (Figure 3A). Some residual activity remained after MNase treatment for the primer d(GT₂G₄)r(U₂G₃) in the reactions shown in Figure 3A, due to the efficient elongation of this primer and incomplete MNase treatment (see also Figure 4A for

Fig. 2. Titration of rUTP, dUTP or TTP in telomerase reactions. The indicated concentrations of rUTP, dUTP or TTP were added to assays in the presence of 0.6 μM [^{32}P]dGTP. A 1 μM concentration of dG₂(G₃T₂G)₃ primer was used. Reactions contained additional MgCl₂ to compensate for nucleotide titrations, as indicated in Materials and methods. (A) Product was precipitated and analyzed by electrophoresis on a 10.5% acrylamide, 7 M urea gel. (B) Product from reactions at the indicated nucleotide concentrations was spotted on DE-81 paper, washed, and quantitated. Scale is in c.p.m. $\circ$, rUTP reactions; Δ , dUTP reactions; $\square$, TTP reactions.

MNase sensitivity). The RNA-containing primers were elongated at concentrations between 10 nM and 10 μ M (data not shown). Over this range, all primers except

d(GT₂G₄)r(U₂G₃) demonstrated no more than a 2-fold increase in product synthesis. In contrast, the chimeric primer d(GT₂G₄)r(U₂G₃), as well as the DNA primers of

Fig. 3. Elongation of RNA and chimeric RNA-DNA primers. (A) The indicated 12- and 18-nt primers were elongated at concentrations of 0.1, 1 or 10 μ M. Extract was either mock-treated (– MNase lanes) or treated (+ MNase lanes) with micrococcal nuclease before inactivation of the nuclease and addition of primer to reactions (see Materials and methods). Product DNA was precipitated and analyzed by electrophoresis on a 12% acrylamide, 7 M urea gel. The products of elongation indicated by arrowheads migrate at the position of primer +1 nucleotide. (B) Aliquots of a reaction containing 1 μ M $r(G_3U_2G)_3$ were removed at the indicated times into stop solution containing EDTA. Product DNA was precipitated and analyzed by electrophoresis on a 12% acrylamide, 7 M urea gel.

equivalent length and sequence to the RNA-containing primers, exhibited a 5- to 10-fold increase in product over this range (data not shown). Figure 3A illustrates this difference in primer concentration dependence with three primer concentrations: 0.1, 1 and 10 μ M. Only a slight shift in the pattern of product DNA was observed with increasing primer concentration for $r(GU_2G_3)_2$, $d(G_3T_2G_4)r(U_2G_4U_2G)$ and $r(G_3U_2G)_3$. Product synthesis was already maximal at 0.1 μ M primer, which is 10-fold less than the saturating concentration of the equivalent DNA primer (Figure 3A). Precise quantitation of the primer concentration dependence of product synthesis for these three primers was unreliable due to the low product yield.

RNA-containing primers of the appropriate permutation (namely, ending with the first guanosine of the $d(TTG\text{---})$ telomeric repeat) were susceptible to the nucleolytic cleavage reaction catalyzed by telomerase (Collins and Greider, 1993). Primers $r(G_3U_2G)_3$ or $d(G_3T_2G_4)r(U_2G_4U_2G)$ could be shortened by at least the primer 3' rG residue: in Figure 3A, these primers promoted synthesis of a high percentage of product at the size of primer itself, resulting from removal of the 3' primer residue and addition of radiolabeled dGTP (Collins and Greider, 1993; products at primer +1 nt are indicated by arrowheads for all the primers in Figure 3A). Although the terminal guanosine of an RNA-containing primer can be removed by telomerase-mediated cleavage, most RNA residues of the primers used in this study are not removed before elongation. This is evident from the offset (slower) migration of products from primers containing RNA nucleotides relative to products from size- and sequence-matched DNA primers.

For all of these RNA and chimeric RNA-DNA primers, the maximal amount of product synthesis was significantly reduced compared with the equivalent DNA primers (see Figure 4 and below). To determine if the reduced level of product synthesis resulted from inactivation of telomerase early in an elongation reaction, perhaps due to formation of an RNA template-RNA primer hybrid, we performed a time-course of elongation. Elongation of $r(G_3U_2G)_3$ (Figure 3B) and the other RNA-containing primers (data not shown) demonstrated that product continued to increase from the first minute of the reaction for >1 h. Thus, telomerase remains active in elongation reactions of RNA-containing primers.

Competition of RNA and chimeric RNA-DNA primers for elongation of DNA

Because RNA-containing primers were optimally elongated at lower concentration than equivalent primers of DNA yet stimulated the synthesis of less product, we reasoned that RNA-containing primers might be inhibitors of a DNA primer elongation reaction. Due to differences in migration of the RNA-containing primers on denaturing gels and differences in primer sequence permutation, the products resulting from elongation of RNA or RNA-DNA chimeric primers could be readily distinguished from products of elongation of a DNA primer in reactions containing both substrates.

We tested the ability of RNA-containing primers to compete for elongation of an optimal, 18-nt telomeric DNA primer, $d(G_3T_2G)_3$ (Figure 4). The RNA-containing

Fig. 4. Elongation of d(G₃T₂G)₃ in the absence or presence of the RNA and chimeric RNA–DNA primers. Reactions were precipitated and electrophoresed on an 11% acrylamide, 7 M urea gel. (A) and (B) were from the same gel and exposure. Arrowheads indicate the migration of the predominant product from elongation of the 12-nt chimeric primer d(GT₂G₄)r(U₂G₃). (A) Elongation of RNA and chimeric RNA–DNA primers alone, at the concentrations used in (B). Reactions were mock-treated (– MNase) or treated (+ MNase) with micrococcal nuclease before inactivation of the nuclease and addition of primer to reactions. Primers were assayed at 0.5 or 5 μM concentration. (B) Elongation of d(G₃T₂G)₃ in the absence (lanes 1,2) or presence of competitor RNA and chimeric RNA–DNA primers. Reactions were done in duplicate and loaded in adjacent gel lanes. All reactions contained 0.5 μM d(G₃T₂G)₃ with the indicated competitor RNA or chimeric RNA–DNA primer at 0.5 or 5 μM concentration.

primers were added as competitors at concentrations equal to the DNA primer (0.5 μM) or in 10-fold excess (5 μM). Elongation of the competitor RNA primers alone at these concentrations is shown in Figure 4A, compared with elongation of the RNA-containing primer and the DNA primer together in Figure 4B. Product synthesis with the RNA-containing primers alone was weak relative to DNA-primed synthesis but was sensitive to MNase pre-treatment of telomerase (Figure 4A, for example see arrowhead indicating product of primer in lanes 1–3). In competitions with the optimal 18-nt DNA primer, differences in inhibition efficiency of the RNA and RNA–DNA chimeric primers were evident (Figure 4B). The chimeric primer

d(GT₂G₄)r(U₂G₃), containing only 5 nt of RNA, was elongated at both 0.5 and 5 μM concentrations either alone (Figure 4A, lanes 1–2; predominant product at the position of the arrowhead) or in the presence of d(G₃T₂G)₃ (Figure 4B, lanes 3–6; predominant product at the position of the arrowhead). However, this primer only weakly inhibited synthesis primed by d(G₃T₂G)₃ relative to the other competitor primers. For the remaining RNA and chimeric RNA–DNA primers, incubation with a concentration equal to that of d(G₃T₂G)₃ (0.5 μM) inhibited product synthesis (Figure 4B). At 10-fold greater primer concentration, only r(G₃U₂G)₃ incompletely inhibited elongation of the DNA primer (although inhibition was much greater

than observed with primer $d(GT_2G_4)r(U_2G_3)$ described above). Based on this assay, the relative order of inhibition efficiency was $r(GU_2G_3)_2 > d(G_3T_2G_4)r(U_2G_4U_2G) > r(G_3U_2G)_3 > d(GT_2G_4)r(U_2G_3)$. We conclude that RNA and chimeric RNA–DNA primers are strong competitors for elongation of telomeric DNA primers.

Competition of RNA and chimeric RNA–DNA primers for telomeric primer binding to the p95 subunit of telomerase

We have previously developed a cross-linking assay which reflects specific primer binding to the p95 protein subunit of telomerase (Collins *et al.*, 1995). Characterization of the specificity of the cross-link suggested that it represents binding of primers to a template-independent site, possibly the anchor site required for processive elongation. Previous experiments demonstrated that telomeric repeat DNAs, added as unlabeled competitors to cross-linking reactions, inhibited the cross-linking of a radiolabeled telomeric DNA probe to p95. However, oligonucleotides complementary to the telomerase RNA and other non-telomeric oligonucleotides did not compete for the cross-linking of p95. Although ribosomal RNAs did not compete for p95 cross-linking (5S, 16S and 28S; Collins *et al.*, 1995), we reasoned that telomeric RNA and RNA–DNA chimeric primers might compete for cross-linking, if competition for elongation was due in part to a competition for binding at the anchor site. We therefore tested the ability of the RNA and RNA–DNA chimeric primers to compete for the p95 cross-link, in comparison with competition by telomeric DNA primers of the same length and sequence (Figure 5). The 12-nt telomeric DNA $d(GT_2G_3)_2$ (lanes 8 and 9, Figure 5) competed poorly relative to the 18-nt telomeric DNA $d(G_3T_2G)_3$ (lanes 2 and 3, Figure 5), as reported previously (Collins *et al.*, 1995) and as expected from the relative elongation efficiencies of these two primers. The RNA and RNA–DNA chimeric primers (lanes 4–7 and 10–13, Figure 5) all competed for the p95 cross-link better than the 12-nt telomeric DNA primer but less effectively than the 18-nt DNA primer. Thus, the RNA-containing primers do compete for binding to telomerase p95. However, the efficiency of competition for cross-linking to p95 does not reflect the efficiency of competition for the elongation of telomeric DNAs. Other contaminants of this telomerase preparation which cross-link to telomeric DNA were preferentially competed by any of the RNAs relative to either DNA; this may be the result of telomeric primer cross-linking to other RNPs in some telomerase preparations (such as 7SL; data not shown). These contaminant cross-links do not co-purify with telomerase and do not demonstrate telomeric DNA-specific competition (Collins *et al.*, 1995).

Mapping of primer regions required for inhibition of DNA primer elongation

Because inhibition of cross-linking observed above was not proportional to the inhibition of elongation of DNA primers by the RNA-containing primers, it seemed unlikely that inhibition resulted predominantly from enhanced binding of the 5' end of RNA-containing primers at the anchor site. Thus, we focused our attention at the primer 3' end. Four primers were synthesized of the same

Fig. 5. Competition of RNA, RNA–DNA and DNA primers for cross-linking to telomerase p95. Telomerase was subject to the cross-linking of p95 by a radiolabeled, iodouracil-substituted, 18-nt telomeric primer DNA in the presence or absence of the indicated unlabeled competitors (see Materials and methods). Wedges indicate increasing concentration of competitor, either 2 or 8 μ M for 18-nt primers or 1 or 4 μ M for 12-nt primers, with 0.5 μ M radiolabeled substrate. '0' indicates no competitor added. The arrow labeled '95 kD' indicates the migration of the 95 kDa cross-link specific to telomerase p95, determined from migration of molecular weight markers on the silver-stained gel. The other labeled species result from impurities in this telomerase preparation.

length and sequence as the previous 18-nt primers, with substitution of RNA at the 3' end for primers $d(G_3T_2G)_2G_3r(U_2G)$ and $d(G_3T_2G)_2r(G_3U_2G)$ or substitution of RNA at the 5' end for primers $r(G_3U_2G)_2G_3d(T_2G)$ and $r(G_3U_2G)_2d(G_3T_2G)$. The results from elongation of these RNA-containing primers are shown in Figure 6A, and results from elongation of the 18-nt DNA primer $d(T_2G_4)_3$ in the presence of these primers are shown in Figure 6B. Only the primers $d(G_3T_2G)_2G_3r(U_2G)$ and $r(G_3U_2G)_2d(G_3T_2G)$ demonstrated an increase in product synthesis with increasing primer concentration in the range of 0.05–5 μ M (Figure 6A, lanes 1–3).

As observed previously with the 12- and 18-nt RNA-containing primers, the primers that were optimally elongated at the lowest concentrations promoted the greatest inhibition of elongation of a DNA primer (Figure 6B). Elongation of the primer $d(T_2G_4)_3$ was 3 nt offset in the pattern of product with regard to the predominantly DNA-containing primer $d(G_3T_2G)_2G_3r(U_2G)$ (compare lanes 1–2 with lanes 5–6 of Figure 6B). The relative inhibition of DNA elongation by this set of RNA-containing primers followed the order $d(G_3T_2G)_2G_3r(U_2G) < r(G_3U_2G)_2d(G_3T_2G) < r(G_3U_2G)_2G_3d(T_2G) < d(G_3T_2G)_2r(G_3U_2G)$. Of the primers assayed, $d(G_3T_2G)_2r(G_3U_2G)$ was the most efficient inhibitor of DNA elongation (Figure 6B, lanes 7–10). The inhibition with this chimeric oligonucleotide was comparable with or better than the previously assayed primers with 9–12 nt of RNA at the primer 3' end (compare Figure 6B with Figure 4B). The efficient inhibition and weak elongation of this primer relative to the weak inhibition and efficient elongation of a primer with only three fewer ribonucleotides [$d(G_3T_2G)_2G_3r(U_2G)$; lanes

Fig. 6. Effects of 3' end sequences of chimeric RNA-DNA primers. Reactions were precipitated and electrophoresed on an 8% acrylamide, 7 M urea gel. (A) and (B) were from the same gel, but the autoradiographic exposure of (A) was approximately three times longer than of (B). This was necessary to show the products from elongation of all four primers in (A) and the shift in product DNA pattern in (B), lanes 1-6 (see text). (A) Elongation of chimeric primers alone, at 0.05, 0.5 and 5 μ M concentrations. (B) Elongation of d(T₂G₄)₃ in the absence (lanes 1,2) or presence of competitor chimeric primers. Reactions were done in duplicate and loaded in adjacent lanes. Part of one of the duplicate reactions of r(G₃U₂G)₂G₃d(T₂G) at 0.5 μ M concentration was lost (lane 12). All reactions contained 0.5 μ M d(T₂G₄)₃ with the indicated competitor chimeric primer at 0.5 or 5 μ M concentration.

1-3 Figure 6A, lanes 3-6 Figure 6B] suggests that a critical region for these effects is 4-6 nt from the primer 3' end. The primer r(G₃U₂G)₂G₃d(T₂G) also showed relatively efficient inhibition and weak elongation (lanes 7-9 Figure 6A, lanes 11-14 Figure 6B). Thus, efficient

inhibition did not require the presence of ribonucleotides in the terminal 3 nt of the primer. When only the region 4-6 nt from the 3' end of the primer r(G₃U₂G)₂G₃d(T₂G) was substituted with DNA, generating the primer r(G₃U₂G)₂d(G₃T₂G), elongation improved and inhibition

weakened (lanes 10–12 Figure 6A, lanes 15–18 Figure 6B). These results suggest that the most critical region for RNA substitution is near, but not at, a primer 3' end.

These and previous results suggest three regions of sensitivity to RNA substitution in a primer. In region 1 (primer 5' end; more than 6–7 nt from the 3' end), RNA substitution decreased interaction of a primer with telomerase. Primers with RNA in this region were somewhat reduced in their ability to compete for elongation of a DNA primer. In region 2 (4–7 nt from the primer 3' end), RNA substitution had the most dramatic effect. Primers with RNA substitution in this region were the most effective competitors of DNA primer elongation and were themselves elongated inefficiently but optimally at low primer concentration. In region 3, (1–3 nt from the primer 3' end), RNA substitution of this region alone did not appear to alter significantly the interaction of primers with telomerase. The borders of these regions relative to the primer 3' end are likely to vary, depending on the primer permutation and sequence.

Discussion

rNTPs and dNTPs as substrates

We demonstrate the incorporation of rGTP and elongation of RNA by telomerase, an RNA-dependent DNA polymerase enzyme. The DNA-dependent DNA polymerase PolI from *Escherichia coli* has also been shown to misincorporate ribonucleotides at a relatively high frequency (for a review, see Kornberg and Baker, 1992). However, significant misincorporation occurs only under atypical reaction conditions, in the presence of non-native divalent cations. In this context, the specificity of telomerase for incorporation of rGTP relative to dGTP is surprisingly low, corresponding to only a ~100-fold difference in the nucleotide concentration required for half-maximal activity. Telomerase also exhibited a bias in estimated K_m for dGTP relative to the native substrate TTP, requiring 10-fold less dGTP than TTP for half-maximal activity as noted previously (Lee and Blackburn, 1993). Telomerase activity was inhibited non-specifically by high nucleotide concentrations of ~1–10 mM. Thus, the failure of telomerase to incorporate significant rUTP may result from the combination of a lower affinity for pyrimidine versus purine, a lower affinity for ribonucleotide versus deoxynucleotide, and a non-specific inhibition at millimolar nucleotide concentrations.

In the presence of [³²P]TTP alone, nucleotide addition to primer dG₂(G₃T₂G)₃ required cleavage of the primer 3' end. After removal of primer 3' residues, TTP incorporation occurred at the correctly templated positions (Figure 2A). However, in the presence of dGTP alone, telomerase was able to elongate a primer by significantly more than four guanosine residues (Figure 1A). Thus, telomerase can apparently ignore instruction by the template to synthesize telomeric repeats and act instead to synthesize poly-d(G). Some RNA-dependent RNA polymerases have also been observed to incorporate a non-templated series of guanosine nucleotides (Ishihama *et al.*, 1986). Even in the presence of [³²P]TTP, high concentrations of dGTP altered the pattern of product DNA, resulting in synthesis of a 1-nt ladder of short products. This product must contain some TTP to become radiolabeled. By analogy to

the 1-nt product ladder obtained in the presence of dGTP alone, it is likely that products from reactions with [³²P]TTP and high concentrations of dGTP resulted from a mixture of initial addition of TTP or a telomeric repeat and subsequent poly-d(G) addition activity. Alternatively, high dGTP concentration could affect the pattern of product DNA synthesis, for example by altering product dissociation, without affecting the sequence of product DNAs.

RNA and DNA as primers

We observed that primers composed entirely or partly of RNA were elongated by telomerase. The amount of product elongation decreased with d(G₃T₂G)₂G₃r(U₂G) > d(GT₂G₄)r(U₂G₃) ~ r(G₃U₂G)₂d(G₃T₂G) > d(G₃T₂G₄)r(U₂G₄U₂G) ~ r(G₃U₂G)₃ ~ r(G₃U₂G)₂G₃d(T₂G) ~ d(G₃T₂G)₂r(G₃U₂G) ~ r(GU₂G₃)₂. In this order, synthesis decreased both in processivity and in intensity of products from addition of only one repeat. When these primers were used in competition with DNA primers for elongation, inhibition of DNA elongation was correlated roughly inversely with amount of product synthesis of the RNA-containing primers themselves: d(G₃T₂G)₂G₃r(U₂G) < d(GT₂G₄)r(U₂G₃) < r(G₃U₂G)₂d(G₃T₂G) < r(G₃U₂G)₃ < r(G₃U₂G)₂G₃d(T₂G) < d(G₃T₂G₄)r(U₂G₄U₂G) < r(GU₂G₃)₂ < d(G₃T₂G)₂r(G₃U₂G). The higher primer concentration required for optimal elongation of primers d(GT₂G₄)r(U₂G₃), d(G₃T₂G)₂G₃r(U₂G) and r(G₃U₂G)₂d(G₃T₂G) correlated with a weaker inhibition of DNA elongation at a given primer concentration, relative to the other RNA-containing primers.

Binding to telomerase p95, reflected by competition of cross-linking, increased with the order d(GT₂G₃)₂ < d(GT₂G₄)r(U₂G₃) ~ r(GU₂G₃)₂ < r(G₃U₂G)₃ ~ d(G₃T₂G₄)r(U₂G₄U₂G) < d(G₃T₂G)₃. For the 12-nt primers, 5 nt of RNA at the primer 3' end improved binding relative to a DNA primer by the same extent whether the primer 5' end was composed of RNA or DNA. For the 18-nt primers, 9 nt of RNA at the primer 3' end decreased binding relative to a DNA primer by the same extent whether the primer 5' end was composed of RNA or DNA. Thus, 12-nt primers with the 3' end r(U₂G₃) bound better than DNA but 18-nt primers with the 3' end r(U₂G₄U₂G) bound worse than DNA. This suggests that different regions of a primer have specific interactions with telomerase which are uniquely influenced by RNA substitution. With regard to p95 cross-linking, these effects were relatively small. As expected from the processive elongation of product with incorporated TTP and rGTP (Figure 1A), RNA-containing primers did compete for cross-linking of a DNA substrate at concentrations comparable with DNA primers. Thus, it is likely that RNA can bind at the telomerase anchor site required for processive elongation.

These results suggest a model in which stronger interaction of a region near the 3' end of RNA-containing primers with the telomerase enzyme decreases the amount and processivity of product synthesis. This could occur by enhanced interaction of RNA-containing primers with the telomerase RNA or with the telomerase proteins. It may be possible to distinguish between these alternatives by the use of modified nucleotides: some modifications may interfere differently with the two types of interactions. An RNA:RNA duplex formed by annealing of an RNA

region of primer and the RNA template would be predicted to have more thermodynamic stability than the cognate DNA:RNA hybrid. This structure could enhance binding and reduce elongation. Perhaps dissociation of a primer bound in this manner occurs only after slow addition of dNTP(s). However, significant inhibition was observed with the primers $r(G_3U_2G)_2G_3d(T_2G)$ and $r(G_3U_2G)_2d(G_3T_2G)$, and the primer $d(GT_2G_4)r(U_2G_3)$ was elongated processively. Thus, more than just propensity for formation of RNA:RNA duplex near the primer 3' end is required to explain the inefficient elongation of RNA-containing primers and their efficient inhibition of DNA elongation.

Synthesis and priming of RNA: telomerase function or evolution?

In vivo, the only known function of telomerase is the extension of the G-rich strand of telomeric repeats. Therefore, why telomerase can recognize primers of RNA and incorporate ribonucleotides is not obvious. One explanation is that telomerase may have function(s) in addition to synthesis of telomeric DNA which require RNA or rNTP recognition. However, deletion of a yeast telomerase RNA revealed no evidence for essential secondary functions of telomerase (Singer and Gottschling, 1994). In addition, telomerase may possess the ability to recognize and utilize RNA and rNTP substrates as the remnant of evolution from an RNA-dependent RNA polymerase. RNA-dependent RNA polymerases and telomerase both utilize a single-stranded template and synthesize single-stranded product; only minor changes in nucleotide and primer specificity would have been required for an interconversion of function. Some sequence similarity of telomerase p95 with the active site of RNA-dependent RNA polymerases has been noted (Collins *et al.*, 1995). RNA-dependent RNA polymerase(s) have been suggested to be the precursor(s) of modern-day polymerases (Lazcano *et al.*, 1988; Poch *et al.*, 1989). Thus, telomerase may have evolved from an ancestral RNA-dependent RNA-polymerase rather than a DNA- or RNA-dependent DNA polymerase enzyme.

Materials and methods

Purification of telomerase

For rNTP titrations, telomerase was purified largely as described (Collins and Greider, 1993). In brief, *Tetrahymena thermophila* were grown to a density of $2\text{--}4 \times 10^5/\text{ml}$, harvested, placed in starvation media for 18–24 h, and harvested again. Cells were lysed by addition of 0.2% NP-40, and a cytoplasmic extract was recovered after high-speed centrifugation. This extract was enriched for telomerase by chromatography with heparin-agarose and DEAE-agarose. Fractions with peak telomerase activity and RNA were ~100-fold enriched for telomerase when compared with the cytoplasmic extract.

For the remainder of experiments, telomerase was purified as described (Collins *et al.*, 1995). In brief, cytoplasmic extract prepared as above was subjected to chromatography on hydroxylapatite, spermine-agarose, Sepharose CL-6B, phenyl Sepharose and DEAE-agarose before sedimentation in a glycerol gradient. These procedures resulted in purification of telomerase to near homogeneity.

RNA, DNA and chimeric RNA–DNA primers

RNA and chimeric RNA–DNA primers were either obtained from Integrated DNA Technologies Inc. (Coralville, IA; 12-nt primers) or synthesized on an ABI model 380a synthesizer with ribonucleotide phosphoramidites (18-nt primers). DNA primers were synthesized by Operon Technologies (Alameda, CA) or on an ABI synthesizer by standard protocol. For gel purification, RNA or DNA primers were

electrophoresed on ~8–10.5% acrylamide, 7 M urea gels, detected by brief UV shadowing, and excised from the gel. Gel slices were crushed, and primers were eluted with several volumes of DEPC-treated water at 37°C. After desalting and concentration, an aliquot of each primer was end-labeled with T4 polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Sambrook *et al.*, 1989) and electrophoresed on a denaturing acrylamide gel to ensure the purity of full-length primer. The 12-nt primers required two rounds of gel-purification.

Telomerase activity

Telomerase activity was assayed using one-half reaction volume of telomerase diluted to a final concentration within the linear range of enzyme activity (buffer: 20 mM Tris–HCl, pH 8.0, 1 mM MgCl_2 , 10–25% glycerol) and one-half volume of other, pre-mixed assay components. Unless otherwise stated, reactions contained a final concentration of 0.3–0.6 μM $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$, 500 μM TTP and 1× telomerase reaction buffer (50 mM Tris acetate, pH 8.5, 100 mM potassium acetate, 1 mM spermidine, 5 mM β -mercaptoethanol). For the nucleotide titration experiments in Figures 1 and 2, additional MgCl_2 was added to the assay mix, providing a final MgCl_2 concentration of 2.5 mM (nucleotide concentrations of 1 mM or less) or 12.5 mM (nucleotide concentrations of 10 mM). Reactions were incubated at 30°C for 20 min (Figures 1, 2, 4 and 6) or up to 1 h (Figure 3) before addition of 2.5 vol of stop buffer (10 mM Tris–HCl, pH 7.5, 20 mM EDTA). At this point, some reactions (Figures 1B and 2B) were vacuum-blotted to DE-81 paper, washed three times for 10 min each in 0.5 M Na_2HPO_4 , rinsed in distilled water and 95% ethanol, dried, and quantitated by scintillation counting. Other reactions were stopped, extracted with phenol:chloroform, and precipitated with 0.3 vol 2.5 M ammonium acetate, 5 μg tRNA and 4 vol ethanol at –20°C. Product was collected by centrifugation, resuspended in formamide, briefly heated in boiling water, and electrophoresed on 10.5–12% acrylamide, 7 M urea gels. For MNase treatment, telomerase was incubated with 1–3 U MNase (Worthington) per μl extract with 1 mM CaCl_2 at 30°C for 30 min. Digestions were stopped before the activity assay by addition of 3 mM EGTA. For RNase T1 treatment, reactions were adjusted with 2.5 vol of stop solution (50 mM Tris, pH 7.5, 4 mM EDTA) then incubated with or without 2 μl (4000 U) RNase T1 (Gibco BRL) for 15 min at 37°C before phenol:chloroform extraction and precipitation as above.

Cross-linking

Cross-linking reactions were performed and analyzed as described (Collins *et al.*, 1995). In brief, the iodouracil (^3U)-substituted telomeric primer $d(GT_2G_3)_2G^3U_2G_3$ was end-labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by T4 polynucleotide kinase (Sambrook *et al.*, 1989), phenol:chloroform extracted, desalted by NAP-5 column (Pharmacia), and concentrated by Speed-Vac. Purified telomerase was adjusted to 1× reaction buffer (above), and 0.5 μM of the cross-linking substrate were added in the presence or absence of competitor unlabeled primers. These reactions were incubated at 30°C for 10 min, irradiated in a Stratilinker equipped with 312 nm bulbs for 500 mJ, digested with 25 U MNase in 1 mM CaCl_2 at room temperature for 15 min, and denatured by addition of 5× SDS sample buffer supplemented with EDTA and EGTA (5× is 0.3 M Tris–HCl, pH 6.8, 10% SDS, 25% β -mercaptoethanol, 50% glycerol, 50 mM EDTA, 20 mM EGTA). Samples were electrophoresed on 5–15% acrylamide gradient SDS minigels and silver stained to ensure parallel recovery of all reactions. Gels were dried and subjected to autoradiography.

Nucleotides

Both dNTPs and rNTPs were 'ultra-pure' grade from Pharmacia. Any contaminants should be mono- or di-phosphates, with no deoxynucleotide impurities in the chemically synthesized ribonucleotide stocks.

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