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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Telomerase is a ribonucleoprotein complex responsible for the maintenance of the terminal repeats of chromosomes. Specific activation of telomerase in most cancer cells confers these cells with unlimited replicative potential, and consequently promotes tumor growth. Telomerase is therefore an attractive target for anti-cancer therapeutics. Studies of telomerase in a variety of systems have revealed a tightly associated nuclease activity, whose physiologic function and molecular identity remain to be elucidated. We have recently shown that the telomerase complex from the budding yeast *Saccharomyces cerevisiae* also possesses a tightly associated nuclease activity. Consistent with results from other systems, we found that the yeast nuclease is single-strand specific and works via an endonucleolytic mechanism. In addition, the reverse transcriptase activity of telomerase can extend either the 5′ or 3′ fragment following cleavage. These results suggest that telomerase either possesses two distinct active sites for the nuclease and reverse transcriptase activity, or possesses two protomers, each containing a bi-functional active site. Careful analysis of a highly purified N-terminal domain of yeast TERT failed to reveal an autonomous nuclease activity. In addition, we identified a point mutation of TERT that exhibits a relative enhancement of the nuclease activity. This point mutation alters a residue in the vicinity of the reverse transcriptase active site. Taken together, these results suggest that the same active site of telomerase carries out both the polymerization and nucleolytic reaction.

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Table of Contents

Introduction

Telomerase is a ribonucleoprotein complex responsible for the maintenance of the terminal repeats of chromosomes. The absence of telomerase activity appears to limit the replicative potential of normal somatic cells. Conversely, activation of telomerase in cancer cells confers enhanced replicative potential and promotes tumor cell proliferation. Specific inhibition of telomerase in several tumor cell lines has been found to cause telomere shortening, and eventually apoptosis. Telomerase is therefore believed to be a valid target for anti-cancer drug development. Telomerase is a multi-component complex, with a reverse transcriptase activity that is primarily responsible for telomere maintenance. In addition, studies of telomerase in a variety of systems have revealed a tightly associated nuclease activity. However, the physiologic significance and the molecular identity of this nuclease have not been determined.

We have recently shown that the telomerase complex from the genetically tractable budding yeast *Saccharomyces cerevisiae* also possesses a tightly associated nuclease activity. Consistent with results from other systems, we found that the yeast nuclease is single-strand specific and acts through an endonucleolytic mechanism. This proposal seeks to define the identity of the telomerase-associated nuclease, analyze its relationship to the reverse transcriptase polypeptide, and determine its physiologic function in telomere metabolism. The methodology involves expression, purification, and biochemical characterization of individual domains of the telomerase polypeptide in an effort to identify the structural determinant of the nuclease. In addition, conserved amino acid residues in the telomerase polypeptide are being mutated in an effort to abolish the nucleolytic activity. Mutants of telomerase that are specifically defective in the nucleolytic activity can be used to address the physiologic function of the nuclease.

Body

As described in the original proposal, we have identified a nuclease activity in partially purified yeast (*Saccharomyces cerevisiae*) telomerase fractions that can cleave the starting DNA primer. Such an activity has also been observed in telomerase from other fungi and ciliated protozoa (Cohn and Blackburn, 1995; Greene et al., 1998; Lue and Peng, 1997; Melek et al., 1996). This activity appears to be tightly associated with the reverse transcriptase activity of telomerase. In addition, cleavage by the nuclease appears to be influenced by telomerase RNA-primer interactions. However, despite extensive characterization of the nuclease in ciliated protozoa, its identity and physiologic function remain obscure. We reasoned that a detailed investigation of this nuclease in the genetically tractable model organism *S. cerevisiae* may allow delineation of its function and physiologic importance.

Since obtaining funding, we have further characterized the nuclease activity in yeast telomerase fractions and have made several interesting observations that bear on its mechanism and its relationship to the reverse transcriptase activity. **First**, we have shown that the nuclease is tightly associated with the reverse transcriptase polypeptide. Three different affinity tags were fused separately to the reverse transcriptase polypeptide, and the appropriate affinity resins used for telomerase purification. In each case, the nuclease was found to co-purify with the primer extension activity of telomerase. **Second**, we showed that both the primer concentration and the nucleotide concentration can influence the ratio of cleavage to extension, suggesting an interesting interplay between these two reaction pathways in the telomerase complex. **Third**, we showed that primers containing non-telomeric cassettes are preferentially cleaved by the telomerase-associated nuclease, implying an effect of RNA-primer interaction on the cleavage pathway. **Fourth**, we showed by the use of primers containing non-hydrolyzable linkages that the nuclease most likely acts as an endonuclease, and that there appears to be a significant degree of flexibility with respect to cleavage site selection. **Finally**, we showed by the use of primers containing chain terminator nucleotides that following cleavage, the reverse transcriptase activity of telomerase can extend both the 5' and the 3' fragment, implying the presence of more than one active site in the enzyme complex. Overall, these results demonstrate that the nuclease is unlikely to be a non-specific contaminant, and that its further characterization may reveal important aspects of telomerase function and mechanisms. A manuscript describing these observations has been accepted and published by Molecular and Cellular Biology (Appendix 1).

In the original application, we proposed based on analogy with non-LTR retrotransposons, that the telomerase-associated nuclease may be encoded by a separate domain of the reverse transcriptase polypeptide (Luan et al., 1993). Therefore, we attempted to demonstrate a specific nuclease activity for the telomerase reverse transcriptase polypeptide (TERT) (Task 1). However, the TERT protein has proved difficult to express in both *E. coli* and yeast. Thus far we have only been successful in generating an N-terminal stable domain of TERT encompassing the first 160 amino acids. Detailed biochemical analysis failed to reveal an autonomous nuclease in this domain. In a separate study, we identified a point mutation in the reverse transcriptase domain of TERT that, in the context of the telomerase complex, altered the relative efficiency of the cleavage and primer extension activity. The point mutation alters a residue near the α-phosphate of the nucleotide substrate. This result suggests that contrary to our original hypothesis, the nuclease activity may in fact be mediated by the reverse transcriptase active site. Further studies are underway to test this alternative hypothesis.

Task 1. To define the molecular identity of the telomerase-associated nuclease (months 1-18).

- *1.a. Develop expression systems in E. coli, yeast, or Baculovirus for the putative nuclease (months 1-12)*

Initial attempts at expressing full-length yeast TERT were unsuccessful. To identify regions of the telomerase protein that may constitute stable domains (and therefore more amenable to recombinant over-production), we carried out a detailed comparative sequence analysis of all available TERTs using a variety of algorithms. This analysis led to the proposition that TERT consists of a conserved N-terminal extension attached to the C-terminal reverse transcriptase domain. The N-terminal extension of TERT may in turn be composed of four universally conserved motifs that are organized into two domains (Fig. 1). This hypothetical domain structure turns out to be quite consistent with the results of the expression studies as described below. All telomerase polypeptides may therefore have a bi-partite structural organization in their N-terminal extension.

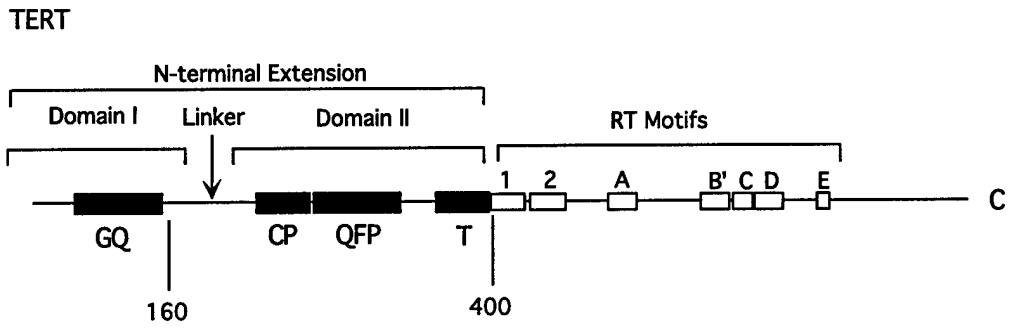

Figure 1. *Schematic illustration of TERT.*
The telomerase reverse transcriptase is an ~1000 kd protein with RT-like motifs located in the C-terminal half. Comparative sequence analysis identified 4 additional conserved motifs located in the N-terminal extension, named GQ, CP, QFP and T. A highly divergent region separating the GQ and the CP motif may constitute a flexible linker that divide the N-terminal extension into two domains.

We used a variety of fusion protein systems to express N-terminal fragments of the yeast TERT (Est2p) in *E. coli*. The fragments that we attempted to over-express included the following: 1-340, 1-304, 1-270, 1-160, 1-100, 1-50 (the numbers designate the amino acid residue numbers in the intact Est2p). Both the glutathione-S-transferase (GST) and maltose-binding protein (MBP) system (Pharmacia and New England Biolab) were tested, with the MBP system yielding consistently higher expression levels.

Interestingly, all fragments larger than 1-160 appear to be sensitive to proteolysis in *E. coli*. For example, the fusion protein consisting of the MBP tag and the Est2p 1-304 fragment (referred to as "MBP-Est2(1-304)p") is apparently unstable in *E. coli*. Following affinity purification over a maltose column, fractions derived from the "MBP-Est2(1-304)p" overproducing strain contained not only the full-length fusion polypeptide, but also several smaller fragments (Appendix 2). These smaller fragments most likely resulted from proteolysis of the Est2 segment *in vivo*, because they still retained the MBP domain, and because the MBP domain on its own was stably expressed in *E. coli* (Appendix 2). Based

on their size, the proteolyzed fragments appear to retain ~160 amino acids of Est2p (Appendix 2). In contrast, all fragments that are smaller than 1-160 can be easily overproduced and purified as a fusion proteins *from E. coli* (Appendix 2). Taken together, our expression studies suggest that the first 1-160 residues of Est2p (encompassing the conserved GQ motif, Fig. 1) comprise a stable, well-folded domain (domain I). The protease-sensitivity of the region immediately C-terminal to residue 160 suggests that this region constitutes an exposed and flexible linker, as hinted by its lack of sequence conservation.

To facilitate further biochemical characterization, we attempted to remove the affinity tag (i.e., the MBP and GST tag) from the Est2p fragments. However, cleavage using the appropriate site-specific proteases designed for the respective systems (i.e., factor Xa for MBP fusions and thrombin for GST fusion) proved to be extremely inefficient, possibly due to steric hindrance. We therefore tested an intein-based expression system that utilizes a small reducing agent to effect cleavage (IMPACT T7 from New England Biolab). With this system, we successfully expressed and purified a significant amount of the Est2p 1-160 fragment in isolation (data not shown).

We have also attempted to express the more C-terminal regions of TERT in *E. coli* using the MBP, GST and intein fusion systems. However, neither domain II of the N-terminal extension (encompassing the CP, QFP and T motifs, Fig. 1) nor the RT domain has proved to be amenable to any of the expression system utilized. Others have shown that domain II of TERT is likely to mediate RNA binding, raising the probability that the domain may not be well-folded or stable in the absence of telomerase RNA. Expression plasmids containing the RT domain were difficult to generate, suggesting that the RT domain in isolation may be toxic to cells.

In summary, we have successfully expressed and purified an N-terminal domain of TERT for biochemical characterizations. In addition, the expression studies provided physical support for a bi-partite structural organization for all TERTs in their N-terminal region. A manuscript incorporating these results has been accepted and published by Molecular and Cellular Biology (Appendix 2).

- *1.b. Test purified recombinant proteins for nuclease activity (months 1-15)*

Because the Est2(1-160)p was the largest N-terminal domain that we can express and purify to homogeneity, we have concentrated on testing this fragment for nuclease activity. Two protein preparations were analyzed in detail: an MBP-Est2(1-160)p fusion and Est2(1-160)p alone (expressed as a fusion protein with an Intein-tag and cleaved from the Intein-tag with DTT). Incubation of 5' end-labeled telomere-like oligonucleotides with both protein preparations resulted in degradation of the DNA. However, the level of protein required was very high, and the MBP tag alone (purified separately employing identical procedures) also mediated DNA cleavage, suggesting that the activity was due to a low-level contaminant (data not shown).

To further investigate the authenticity of the nuclease, we fused an additional His_6 tag to the C-terminus of the fusion protein (to make MBP-Est2(1-160)p-His) to allow tandem affinity purification of the domain. We also expressed two additional fusion proteins, MBP-Est2(1-50)p-His and MBP-Est2(50-160)p-His, with the expectation that only one of the sub-regions should possess nuclease activity if the activity is due to Est2p. All three fusion proteins were expressed, purified to near homogeneity, and tested for nuclease activity. Contrary to

our expectations, very high levels of proteins were required for detectable DNA cleavage, and each fragment mediated comparable levels of cleavage, suggesting that the cleavage was due to a contaminating *E. coli* protein. We conclude from this and earlier results that the Est2p 1-160 domain is unlikely to possess an autonomous nuclease, and that the low level of cleavage observed is likely due to a non-specific contaminant.

Even though our study rules out a specific nuclease for the 1-160 fragment, we were able to demonstrate a functionally important nucleic acid binding activity for this domain. This nucleic acid binding activity requires residues 1-50 and exhibits a slight preference for single-stranded over double-stranded nucleic acids. The precise function of this nucleic acid binding activity and its relationship to the reverse transcriptase and nuclease activity of telomerase remains to be determined. A manuscript incorporating these findings have been accepted and published by Molecular and Cellular Biology (Appendix 2).

The lack of nuclease activity for the 1-160 domain is consistent with the idea that the telomerase-associated nuclease may be mediated by domain II of the N-terminal extension or the RT domain. Consistent with this notion, we have identified a point mutation in the RT domain of TERT that, in the context of the telomerase complex, altered the relative efficiency of the cleavage and primer extension activity. This appears to be the first instance of a TERT mutation affecting the relative extent of primer cleavage. The mutation alters a residue that is positioned near the α-phosphate of the nucleotide substrate, suggesting that the nuclease activity may be mediated by the RT domain. A new line of investigation has been initiated to explore this hypothesis (see below).

- *1.c. Characterize the biochemical properties of the cloned nuclease (months 1-18)*

This task is currently on hold pending the identification of a TERT fragment that does possess an intrinsic nuclease activity.

Task 2. and Task 3. To define key residues necessary for the cleavage activity of the nuclease, and determine the biochemical and physiologic role of the nuclease in telomere metabolism (months 19-30), and to characterize the human homologue of the yeast nuclease (months 31-36)

These tasks have been supplanted by the following new task because of findings that are inconsistent with our original hypothesis.

New task: Characterization of the effects of mutations surrounding the reverse transcriptase active-site on the cleavage activity.

In a separate study, we have identified a point mutation in the RT domain of TERT that, in the context of the telomerase complex, altered the relative efficiency of the cleavage and primer extension activity. This appears to be the first instance of a TERT mutation affecting the relative extent of primer cleavage. The mutation (R450K) alters a residue that is positioned near the α-phosphate of the nucleotide substrate (Huang et al., 1998), suggesting that the nuclease activity may be mediated by the reverse transcriptase active site (Fig. 2). A

manuscript incorporating these findings has been accepted and published recently (Appendix 3).

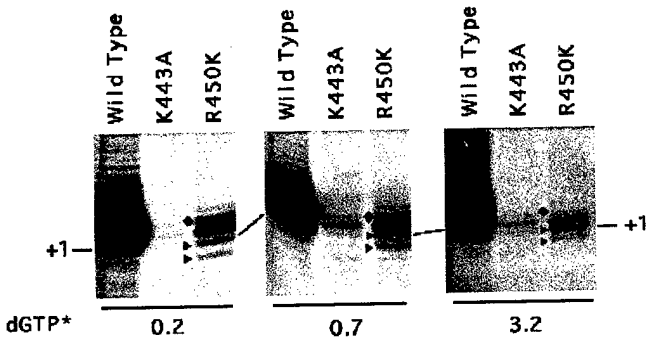

Figure 2. *The R450K mutation specifically enhanced the level of cleavage activity relative to the reverse transcription activity.* The wild type telomerase and the K443A and R450K mutants were tested in primer extension assays in the presence of three different concentrations of labeled dGTP. The positions of the primer+1 product are indicated by lines at the sides of the panels. Cleavage-derived products are marked by triangles. As shown in the figure, the relative amount of cleavage-derived products is greatly increased for the R450K mutant.

One plausible hypothesis that can explain our observation is that under some conditions, an internal phosphodiester linkage of the DNA may be positioned in the reverse transcriptase active site, and that a water molecule rather than the 3' –OH of the DNA primer may act as the nucleophile to effect cleavage. To further explore this hypothesis, we examined the crystal structure of the HIV-1 reverse transcriptase in a complex with template, primer, and nucleotide triphosphates, and identified residues near the putative phosphodiester linkage (e.g., Arg450 and Gln632; Fig. 3). We have created a series of mutations in these residues and are in the process of testing their effects on the nuclease activity. Our hypothesis is that these mutations will either enhance or diminish the relative level of the nuclease activity. In contrast, mutations in residues far away from the phosphodiester linkage should have little effects.

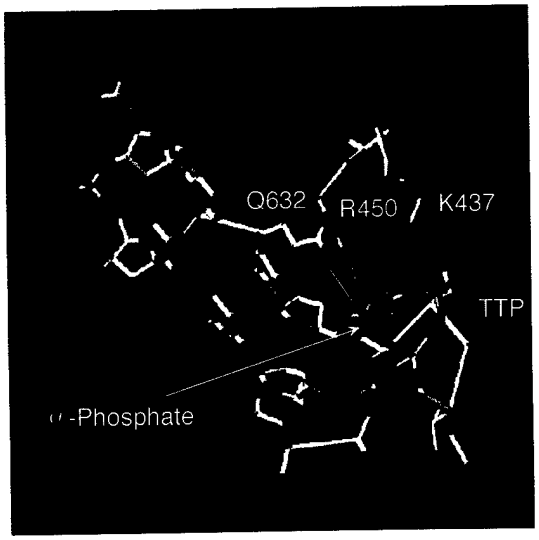

Figure 3. *Residues predicted to affect the cleavage activity of telomerase.* Part of the crystal structure derived from an HIV-1 RT, template, primer, and dTTP complex is shown (Huang et al., 1998). In the cleavage reaction, the α-phosphate of the TTP substrate is postulated to be attacked by a water molecule, resulting in hydrolysis. Residues predicted to influence the cleavage reaction include R450 and Q632 (corresponding to R72 and Q151 in HIV-1 RT). In contrast, the K437 residue, which interact with the γ-phosphate of TTP, is too far away from the active site to have an impact.

The demonstration of the ability of the telomerase reverse transcriptase active site to carry out nucleolytic cleavage will have two major implications. First, because the active site of telomerase is similar to those of conventional reverse transcriptases (e.g., retroviral and retrotransposons reverse transcriptases), such a demonstration would suggest that the conventional enzymes may also be capable of cleavage. Second, based on detailed biochemical analysis, yeast telomerase is likely to possess more than one active site. The correspondence of the reverse transcriptase and nuclease active site would then imply that the enzyme is at least a dimer, as previously suggested (Prescott & Blackburn, 1997).

Key Research Accomplishments

- Demonstrated that the nuclease activity present in yeast telomerase fraction is tightly associated with the reverse transcriptase polypeptide.
- Demonstrated that the yeast telomerase-associated nuclease acts endonucleolytically and is flexible with respect to cleavage site selection.
- Demonstrated that the yeast telomerase complex may contain more than one active site based on the coupling between the nuclease and reverse transcriptase activity.
- Obtained evidence in support of a bi-partite structural organization for the N-terminal region of all telomerase reverse transcriptase.
- Expressed and purified significant amounts of an N-terminal fragment of telomerase reverse transcriptase and showed that it possesses a functionally important nucleic acid binding activity.
- Demonstrated that the most N-terminal domain of yeast TERT is unlikely to possess an autonomous nuclease activity.
- Identified a point mutation in the RT domain of TERT that resulted in a preferential enhancement of nuclease activity.
- Developed new affinity purification protocols for the isolation of the telomerase complex.

Reportable Outcomes

Papers:

1. Niu, H., Xia, J., & Lue, N.F. (2000). Characterization of the interaction between the nuclease and reverse transcriptase activity of the yeast telomerase complex. *Mol. Cell. Biol.* **20,** 6806-6815.

2. Xia, J., Peng, Y., Mian, I.S., & Lue, N.F. (2000). Identification of functionally important domains in the N-terminal region of telomerase reverse transcriptase. *Mol. Cell. Biol.* **20,** 5196-5207.

3. Bosoy, D., Peng, Y., and Lue, N.F. (2001). Functional analysis of the putative finger domain of telomerase reverse transcriptase. *J. Biol. Chem.* **276,** 46305-46312.

Conclusions

We have shown by a number of criteria that the yeast (*Saccharomyces cerevisiae*) telomerase complex possesses a tightly associated nuclease activity that can cleave single-stranded telomeric oligonucleotides *in vitro*. The cleavage reaction can apparently be modulated by primer concentration, nucleotide concentration, and RNA template-primer interaction, suggesting an interestingly interplay between the nuclease and reverse transcriptase activity. Cleavage proceeds by an endonucleolytic mechanism and exhibits a degree of flexibility with respect to site selection. Intriguingly, following cleavage, the reverse transcriptase activity can extend both the 5' and 3' fragment, implying that the telomerase complex possesses more than one active site. These results suggest that further characterization of the nuclease may reveal important aspects of telomerase function and mechanisms.

In an attempt to determine the molecular identity of the nuclease, we have expressed and purified an N-terminal stable domain (1-160) of the yeast telomerase reverse transcriptase polypeptide either alone or as a fusion protein from *E. coli.* Biochemical characterization of the fragment failed to reveal an authentic nuclease for this domain. Instead, a functionally important nucleic acid binding activity was uncovered by our analysis. We also obtained evidence in support of a bi-partite structural organization for the N-terminal region of TERT. This structural organization is consistent with our comparative sequence analysis of telomerase from phylogenetically diverse organisms, and suggests that all TERTs may have such a structural feature.

Because the nuclease appears to be phylogenetically conserved, it may be mediated by some of the conserved amino acid residues. Therefore, in addition to the studies proposed in the original application, we are systematically mutating conserved residues in TERT, and testing the resulting telomerase complex for selective alterations in nuclease activity using the combined cleavage-extension assay (described in Appendix 1). Thus far we have identified a point mutation in the RT domain of TERT (R450K), located near the nucleotide-binding site, that enhances the relative cleavage activity of the complex (Appendix 3). This result suggests that the nuclease may in fact be mediated by the reverse transcriptase active site. To further explore this hypothesis, we are analyzing an extensive set of mutations near the reverse transcriptase active site for possible effects in nuclease activity. Demonstrating the equivalence of the two active sites would have major implications for telomerase structure and function.

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Appendices

Papers and Manuscripts:

1. Niu, H., Xia, J., & Lue, N.F. (2000). Characterization of the interaction between the nuclease and reverse transcriptase activity of the yeast telomerase complex. *Mol. Cell. Biol.* **20,** 6806-6815.

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Characterization of the Interaction between the Nuclease and Reverse Transcriptase Activity of the Yeast Telomerase Complex

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Telomerase is a ribonucleoprotein that mediates extension of the dG-rich strand of telomeres in most eukaryotes. Like telomerase derived from ciliated protozoa, yeast telomerase is found to possess a tightly associated endonuclease activity that copurifies with the polymerization activity over different affinity-chromatographic steps. As is the case for ciliate telomerase, primers containing sequences that are not complementary to the RNA template can be efficiently cleaved by the yeast enzyme. More interestingly, we found that for the yeast enzyme, cleavage site selection is not stringent, since blocking cleavage at one site by the introduction of a nonhydrolyzable linkage can lead to the utilization of other sites. In addition, the reverse transcriptase activity of yeast telomerase can extend either the 5′- or 3′-end fragment following cleavage. Two general models that are consistent with the biochemical properties of the enzyme are presented: one model postulates two distinct active sites for the nuclease and reverse transcriptase, and the other invokes a multimeric enzyme with each protomer containing a single active site capable of mediating both cleavage and extension.

Telomerase is a ribonucleoprotein that is responsible for maintaining the terminal repeats of telomeres in most organisms (1, 2, 28, 37). It acts as an unusual reverse transcriptase (RT), using a small segment of an integral RNA component as template for the synthesis of the dG-rich strand of telomeres (11, 12). DNA synthesis by telomerase in vitro is primed by oligonucleotides with telomere-like sequences. Depending on the source, telomerase in vitro can act either processively, adding many copies of a repeat without dissociating, or nonprocessively, completing only one telomeric repeat (13, 29, 31).

Telomerase activity has been detected in a wide range of organisms, including protozoa (2), yeasts (4, 17, 18, 20, 35), mice (31), *Xenopus laevis* (22), and humans (25). Genes encoding the RNA and RT subunit of the enzyme complex have also been cloned for many known telomerases (2, 3, 5, 8, 16, 18, 24, 26, 34). In addition, both biochemical and genetic studies point to the existence of additional protein subunits of telomerase, whose functions remain to be elucidated (7, 9, 15, 19, 27).

A telomerase-associated nuclease has been identified in *Tetrahymena thermophila*, *Euplotes crassus*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* (4, 6, 10, 20, 21, 23, 29). In the case of *Tetrahymena* telomerase, the associated nuclease has been found to remove one or several terminal primer nucleotides prior to polymerization. Enzyme reconstituted in rabbit reticulocyte lysates with p133 (the RT subunit) and telomerase RNA retains cleavage activity, suggesting that the nuclease resides in one of these two components (5). The nuclease from *E. crassus* has been thoroughly characterized using a coupled cleavage-elongation assay (10, 23), which revealed the following salient features: (i) cleavage proceeds by an endonucleolytic mechanism, (ii) DNA fragments from the 3′ end can be eliminated prior to elongation of the primer by telomerase, (iii) long stretches of preferably nontelomeric sequences can be removed by the nuclease, (iv) cleavage occurs preferentially but not exclusively at the junction of match-mismatch between the primer and the RNA template, (v) the junction of match-mismatch between the primer and the RNA template can be positioned at various locations along the RNA template to effect cleavage, and (vi) primers bearing nontelomeric sequences at the 5′ end are preferentially cleaved. While not as thoroughly studied, the nuclease from other organisms exhibits properties consistent with those displayed by the *Tetrahymena* and *E. crassus* enzymes. For example, both primer-template mismatch and the presence of nontelomeric sequences at the 5′ end have been found to stimulate cleavage by the yeast telomerase-associated nuclease (21, 29).

Various functions have been suggested for the telomerase-associated endonuclease. For example, the combined cleavage and elongation activity may be useful in the de novo formation of telomeres during macronuclear development in ciliated protozoa (23). Alternatively, cleavage may serve a proofreading function given that nontelomeric sequences appear preferentially removed (10, 23). In addition, by analogy with DNA-dependent RNA polymerases, cleavages may allow an elongation-incompetent telomerase to re-engage the 3′ end of the primer prior to extension (5).

In this study, we characterized the *Saccharomyces cerevisiae* telomerase-associated nuclease in greater detail and found that it shares many properties that have been ascribed to the ciliate enzymes. For example, yeast cleavage activity is tightly associated with the polymerization activity. In addition, primers with sequences that are noncomplementary to the RNA template appear to be relatively efficient substrate for cleavage by yeast telomerase. The yeast nuclease also appears to act through an endonucleolytic mechanism. More surprisingly, we found that following cleavage, either one of the fragments

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generated by the yeast nuclease (the 5′ and the 3′ fragments) can be extended by the polymerization activity of telomerase. This result is not easily rationalized in terms of a monomeric enzyme containing a single nuclease-polymerase active site. Two models that are compatible with all of our biochemical observations are presented in the Discussion.

MATERIALS AND METHODS

Yeast strains, media, buffers, and the preparation of yeast telomerase. JX-M3 is a haploid yeast strain identical to W303a except that the EST2 gene in the strain was fused at its C terminus to a Myc_3 epitope tag using a PCR recombination method (35). JX-MH19 contains an EST2 gene whose C terminus is fused to both a Myc_3 epitope tag and a His_6 tag. JX-proA contains an EST2 gene with, in addition to the Myc and His tags, two copies of the immunoglobulin G (IgG) binding domain from protein A. The construction of these strains will be described in detail elsewhere.

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, and 1.5 mM dithiothreitol (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 millimolar concentration of sodium acetate specified by the number in parentheses. The following protease inhibitors were included in all buffers: 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 2 μg of pepstatin A per ml, and 1 μg of leupeptin per ml.

Purification of yeast telomerase. For preparation of whole-cell extracts, the yeast strains DG338 (a gift of D. Garfinkel, National Cancer Institute), W303a, JX-M3, JX-MH19, or JX-proA was grown in YPD medium, lysed in TMG-15(0) buffer, and the lysates were clarified by high-speed centrifugation as previously described (4, 21). To obtain active telomerase, whole-cell extracts were processed over DEAE-agarose columns as previously described (4, 21). For Myc-tag affinity purification, DEAE fractions (10 ml) prepared from the JX-M3 strain were loaded directly onto a 0.5 ml of 9E10 (Myc antibody) column. The column was washed with TMG-10(500) and then TMG-10(500) containing 1 mg of HA.11 (hemagglutinin) peptide per ml at 4°C. Telomerase was then eluted at room temperature with 1.5 ml of TMG-10(500) containing 1 mg of 9E10 (Myc) peptide per ml. The overall recovery of activity was ~10%, while 0.05% of the load had approximately the same amount of total protein as 50% of the purified fraction, based on the staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Because the estimate of the protein concentration is not precise, we did not determine the fold enrichment for telomerase in this immunoaffinity procedure. For metal affinity purification, DEAE fractions (2 ml) prepared from JX-MH19 strain were loaded directly onto a 0.2-ml Ni-nitrilo triacetic acid column (Qiagen). The column was washed successively with TMG-10(500) and TMG-10(500) containing 5 mM imidazole. Active telomerase was then eluted with TMG-10(500) containing 200 and 500 mM imidazole. The majority of telomerase was present in the 200 mM elution. The overall recovery of activity was ~50%, while the purified fraction contained a fraction (ca. 1/20) of the starting protein based on the Bio-Rad Protein Assay (Bio-Rad Laboratories). Thus, we estimate that telomerase is enriched by about 10-fold by the metal affinity procedure. For the protein A-tag-based purification, a DEAE fraction from JX-proA (100 μl) was directly incubated with 5 μl of IgG-Sepharose beads at 4°C with gentle rotation for 2 h. The beads were washed multiple times with TMG-10(600) and then assayed for telomerase activity along with the DEAE fraction and the supernatant. More than 95% of the starting protein remained in the supernatant, while the beads contained ~50% of the starting activity. Thus, telomerase was purified more than 10-fold by this IgG affinity procedure.

For multistep purification, the protein A-tagged enzyme was successively fractionated over DEAE, phenyl, heparin, and IgG columns. DEAE chromatography was carried out as previously described (4, 21). Active fractions from the DEAE column were pooled and loaded directly onto a phenyl Sepharose (Pharmacia) column. The column was washed successively with two column volumes each of TMG-10(500) and TMG-10(100), and the activity was eluted with two column volumes of TMG-10(0) plus 1% Triton X-100. Active fractions were pooled and loaded onto an Affi-Gel Heparin (Bio-Rad) column. The column was washed with two column volumes of TMG-10(150), and the activity was eluted with two column volumes of TMG-10(700). Active fractions were then processed over IgG-Sepharose resin as described earlier. The specific activity and the degree of purification were calculated from primer extension activity assays and protein assays with two exceptions. First, because the activity was undetectable in whole-cell extracts, the fold purification for the DEAE column fraction was based on the degree of Est2p enrichment (as determined by Western blotting). Second, because it is not possible to elute telomerase from IgG-Sepharose, we estimated the amount of total protein bound to the beads to be the difference in protein concentration of the heparin fraction before and after binding to IgG beads. For Western analysis of protein A-tagged Est2p, proteins from extracts or DEAE fractions were separated in by SDS–8% PAGE and transferred onto nitrocellulose membrane. Primary anti-protein A antibody (Sigma) and secondary antibody were used at 1:1,000,000 and 1:5,000 dilutions, respectively. Immunoreactive species were visualized using the ProtoBlot system (Promega).

Primer preparation. DNA primers were purchased from GeneLink (Thornwood, N.Y.) and gel purified prior to use in polymerization assays. Crude primers were dissolved in distilled H_2O at 1 mg/ml and fractionated on a 16% denaturing polyacrylamide gel. Full-length DNA fragments were visualized by ethidium bromide staining and UV transillumination, isolated as small gel slices, and eluted overnight at 37°C with 400 μl of extraction buffer containing 0.1% SDS, 0.3 M sodium acetate, 10 mM magnesium acetate, and 1 mM EDTA. The DNA was recovered from the extraction buffer by ethanol precipitation in the presence of 5 μg of glycogen and resuspended in a suitable volume of water. The concentration of the purified DNA primer was again quantified by PAGE and ethidium bromide staining.

Primers bearing methylphosphonate linkages were also purchased from GeneLink and then gel purified prior to use. Resistance to nuclease was confirmed by using *Escherichia coli* Exonuclease III (New England Biolabs). Primers terminating in dideoxynucleotides were made by treating 500 pmol of DNA primer with 34 U of terminal deoxynucleotide transferase (USB; 17 U/μl) and 833 μM dideoxynucleotides (ddTTP or ddGTP) in 30 μl of total volume containing 1× buffer (USB) at 37°C for 3 h. After phenol-chloroform extraction, full-length DNA was recovered by ethanol precipitation and gel purified as described above.

Coupled cleavage-extension assay. A standard cleavage-extension assay (30 μl) contained 50 mM Tris-HCl (pH 8.0), 1 mM spermidine, 1 mM DTT, 1 mM $MgCl_2$, 130 μM dTTP, 1 to 2 μl of [α-^{32}P]dGTP (3,000 Ci/mmol, 10 μCi/μl), and various amounts of DNA primers and telomerase fractions. Reactions were started by the addition of telomerase fraction to a premixed cocktail consisting of all the other components. Reactions were continued for 1 h at 30°C, and labeled products were processed as described previously (21).

RESULTS

Observation of a nuclease in yeast telomerase fractions. For purification and characterization of yeast telomerase, we utilized a direct primer extension assay (4, 21). Under standard reaction conditions, the yeast enzyme is nonprocessive and gives rise predominantly to a "primer + 3" product (21; Fig. 1A). Interestingly, the use of certain primers in extension assays, especially those consisting of repeats from other organisms, often yielded products that are shorter than the input primer. For example, when *Oxytricha*, human, and *Arabidopsis* repeats are utilized as primers, as much as 20% of the labeled products in the polymerization assays were shorter than the starting primer (Fig. 1A and B). To rule out the possibility that there is excess nonspecific nuclease in the partially purified yeast telomerase fractions, we assembled mock telomerase reactions using the fraction, unlabeled primer, and unlabeled nucleotide triphosphates. Also included in each reaction was a small amount of end-labeled tracer oligonucleotide used to monitor the fate of the input primer. As shown in Fig. 1C, the vast majority of the starting primers are neither shortened nor extended, even in the presence dGTP and dTTP. This result is consistent with the large molar excess of primer over active telomerase as determined by the polymerization assay. No discrete bands can be visualized in the region of the gel presumed to contain the nuclease-derived products, and quantification indicates that this region possesses <2% of the radioactivity present in the full-length bands. These results are quite consistent with earlier observations on the existence of a specific nuclease in yeast telomerase fractions (4, 21, 29).

Since <2% of the input primers were cleaved yet as much as 20% of the extension products were derived from cleaved DNA, telomerase appears to preferentially extend cleaved DNA (by at least 10-fold). This preferential extension can be explained by either a coupling between the telomerase and the nuclease or by short primers being intrinsically superior substrates for telomerase. To address the latter possibility, we assessed the activity of two primers of different lengths (OXYT1 and OXYT2) bearing the *Oxytricha* telomeric repeats at increasing primer concentrations. The shorter primer (OXYT2) was designed to mimic the size of the cleaved but not yet extended DNA derived from the longer primer (OXYT1). As shown in Fig. 2A, the cleavage-derived products

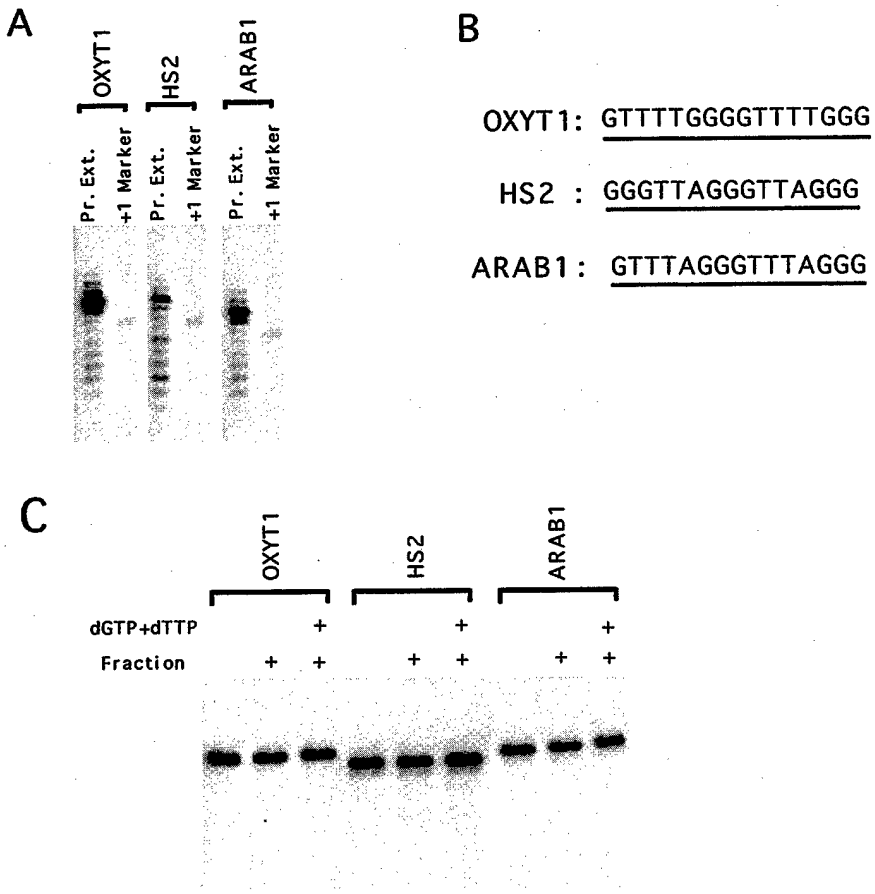

FIG. 1. Yeast telomerase partially purified by DEAE chromatography contains a nuclease activity. (A) Polymerization assays were performed using 5 μM concentrations of various primers and 9 μg of DEAE fractions (Pr.Ext.). Primers labeled by terminal transferase and cordycepin were run alongside the reaction products as size standards (+1 Marker). (B) The sequences of the primers used in panel A. (C) Concentrations (5 μM) of various primers (containing a small amount of labeled tracer) were incubated alone, with telomerase fractions, or with telomerase fraction and deoxynucleotide triphosphates. The DNA was recovered and analyzed by denaturing gel electrophoresis as in standard primer extension assays.

of OXYT1 are indeed similar in size to the direct extension products of OXYT2. Furthermore, at the same molar concentration, OXYT1 and OXYT2 supported a nearly identical amount of DNA synthesis, suggesting that short DNAs are not intrinsically better substrates for yeast telomerase (Fig. 2B). Therefore, a physical or functional coupling between the nuclease and telomerase appears likely.

Affinity-purified telomerase exhibits the same nuclease activity. To determine if the coupling observed between the nuclease and telomerase in the DEAE fraction can be explained by physical association, we further purified yeast telomerase using three different affinity tags (a Myc_3 tag, a His_6 tag, and a protein A tag) and the appropriate chromatographic resins. All three tags were fused to the C terminus of Est2p and had no effect on telomere maintenance or telomerase activity (J. Xia and N. F. Lue, unpublished data). The detailed purification procedures and the estimates for the degrees of enrichment are presented in Materials and Methods. In each case, the affinity-tagged telomerase was first purified over a DEAE column (4, 21). Active enzymes were then adsorbed onto the appropriate affinity columns and either eluted with specific competitors before analysis (for Myc- and His-tagged enzymes) or directly assayed on the resin (for protein A-tagged enzymes). As shown in Fig. 3A, each of the purification procedures resulted in telomerase that was still capable of catalyzing the cleavage-extension reaction on the OXYT1 primer. Furthermore, in each case the fraction of the products that were shorter than the starting primer was similar for both the DEAE and the affinity-purified enzyme (compare lanes 1 and 2, 3 and 4, and 5 and 6). A contaminating activity (or activities) capable of generating labeled high-molecular-weight products is evident in DEAE fractions derived from the Myc- and His-tagged strains (lanes 1 and 3, indicated by brackets to the left of the panels). This activity (or activities) was successfully removed by the affinity procedures.

To further eliminate the possibility of an unrelated, contaminating nuclease, we purified protein A-tagged telomerase using four consecutive chromatographic steps (DEAE, phenyl, heparin, and IgG; see Table 1). The degree of purification was monitored throughout the procedure by protein and activity assays with two exceptions. First, because the activity was undetectable in whole-cell extracts, the fold purification for the DEAE column fraction was based on the degree of Est2p enrichment as determined by Western blotting using anti-protein A antibodies (Fig. 3B). Second, because it is not possible to elute telomerase from IgG-Sepharose, we estimated the amount of total protein bound to the beads to be the difference in protein of the heparin fraction before and after binding to IgG beads. Changes in the polypeptide compositions of the fractions are evident during purification (Fig. 3C). However,

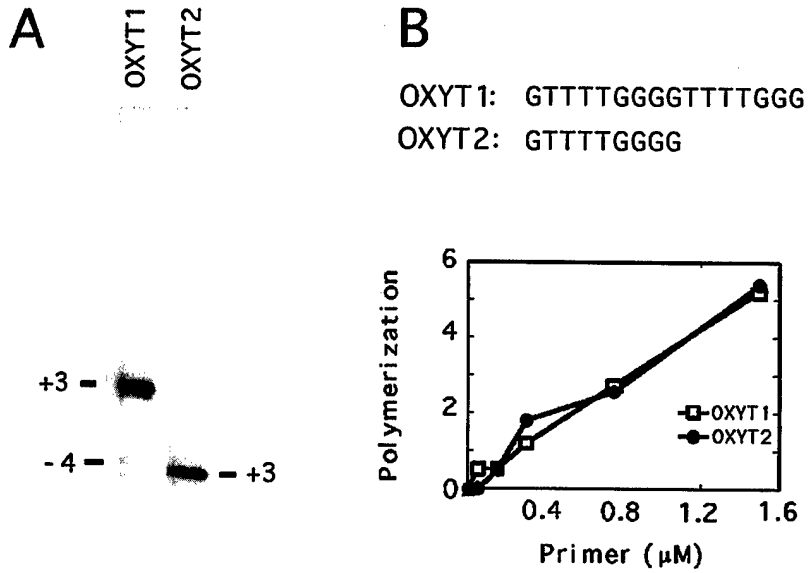

FIG. 2. Long and short heterologous primers are utilized by yeast telomerase at comparable efficiencies. (A) Polymerization assays were performed using 1.5 μM concentrations of either OXYT1 or OXYT2 as the DNA primer and 2.3 μg of DEAE fractions. The locations of the "+3" and "−4" products for OXYT1 and that of the "+3" product for OXYT2 are indicated by horizontal bars. (B) Polymerization assays were carried out using increasing concentrations of either OXYT1 or OXYT2, and the signals derived from direct extension of the primers were quantified and plotted.

because of the low abundance of telomerase in yeast, telomerase-specific polypeptides cannot be identified even after this multistep purification procedure. When tested in the primer extension assay (Fig. 3D), the nuclease-derived products are evident following each chromatographic step, and PhosphorImager analysis indicates that the ratio of cleavage to extension products varied by no more than twofold. Based on these studies, we conclude that cleavage of starting primers by telomerase fractions is unlikely to be due to an unrelated contaminant.

The effects of reaction parameters on telomerase-mediated primer cleavage. To determine if the extent of cleavage is affected by any reaction parameters, we varied the duration and the concentrations of the components of the reaction. Time course experiments indicate that the "direct extension" and "cleavage derived" products accumulate with similar kinetics (38), both being complete within ~15 min. Prolonged incubation does not result in an increase in the relative amount of the cleavage products. Thus, there appears to be little nonspecific nuclease in the fraction that can degrade the labeled products, a finding consistent with the earlier tracer experiment (Fig. 1C). Increasing the salt concentration also did not appreciably affect the ratio of the two classes of products (Figure 4A, lanes 1 to 3). Increasing primer concentration from 3 to 24 μM reduced the relative amount of cleavage products by threefold, suggesting that the direct extension reaction pathway is more favorable at high primer concentrations (Fig. 4A, lanes 4 to 7). More interestingly, when the total dGTP concentration was increased by about 10-fold over the standard reaction (to 2 μM), the cleavage products were almost completely abolished, despite the presence of a significant amount of direct extension products (Fig. 4B, lanes 4 and 5). Thus, the cleavage-extension reaction pathway appears to be favored when the concentration of dGTP is low. Cleavage-derived products were not evident in some published studies on yeast telomerase (18, 19). This discrepancy is most likely due to the use of different primers and higher nucleotide concentrations in these other studies. The concentration of dGTP has been reported to affect the processivity and template utilization of the *Euplotes aediculatus* telomerase (14). Whether these effects of dGTP are related to its ability to influence cleavage remains to be determined.

Primers bearing nontelomeric cassettes are susceptible to cleavage by telomerase. Characterization of the ciliate telomerase-associated nuclease suggests that the cleavage pathway is affected by primer-RNA interactions (10, 21, 23, 29). In general, primer-template mismatches can apparently promote cleavage. In particular, a primer containing a telomeric cassette embedded in nontelomeric sequences was an especially good substrate for cleavage by *Euplotes* telomerase. To determine if the yeast telomerase nuclease has similar properties, we tested yeast telomeric primers bearing nontelomeric cassettes at their 5′ or 3′ end in the polymerization reactions. As shown in Fig. 5, a primer containing either an 8- or a 14-nucleotide (nt) nontelomere cassette at its 5′ end (TEL51 and TEL52) was efficiently extended by telomerase. Such primers also gave rise to a significant amount of "cleavage-derived" products. Interestingly, the size of the cleavage-derived products was similar for these two primers (Fig. 5B, compare lanes 1 and 3). This result suggests that cleavage might have occurred predominantly near the junction of the telomeric and nontelomeric cassettes, thereby releasing telomeric fragments of similar size to be extended by the RT. If this conjecture is true, then telomerase is not only capable of extending the 5′ cleavage fragment, as previously reported, but also the 3′ fragment. This possibility was confirmed in experiments reported in the following sections.

In contrast to primers with 5′ nontelomeric cassettes, primers with the same two cassettes at their 3′ end (TEL106 and TEL107) were poor substrates for telomerase-mediated extension, and few cleavage products could be observed in these reactions. As expected, the 14-nt nontelomere cassette on its own failed to yield any extension product (TEL108).

The yeast telomerase-associated nuclease acts endonucleolytically. The cleavage-derived products for most primers had a nonrandom distribution. For example, for both HS2 and

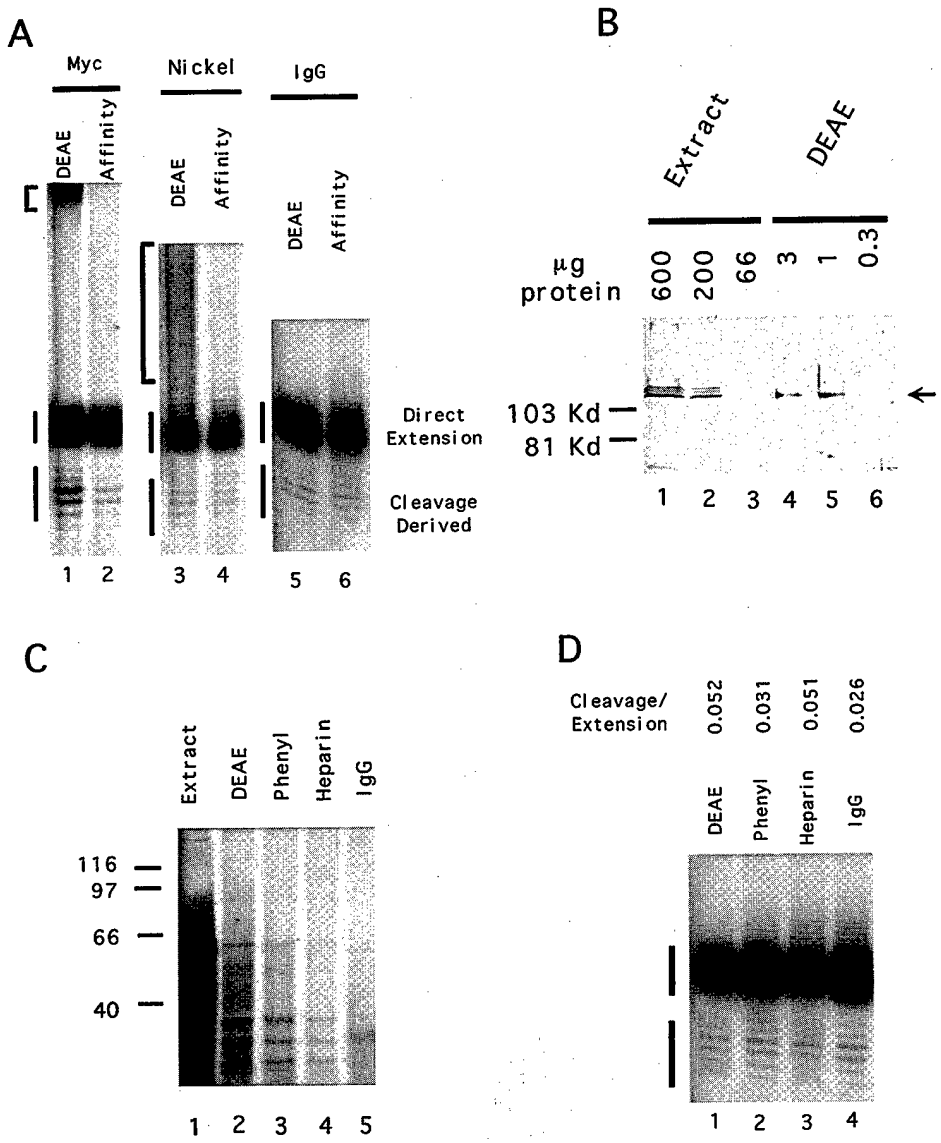

FIG. 3. Affinity-purified yeast telomerase exhibits a similar cleavage activity as that found in DEAE fractions. (A) Polymerization assays were carried out using OXYT1 (1 μg) as the DNA primer and DEAE fraction (DEAE) or affinity-purified telomerase (Affinity) as the source of telomerase. The affinity resin utilized for each purification is indicated at the top. The amount of protein used for each reaction is as follows: lane 1, 3 μg; lane 2, 45 ng; lane 3, 3 μg of protein; lane 4, 0.4 μg of protein; lane 5, 3 μg of protein; lane 4, ~0.15 μg of protein. "Direct extension" or "cleavage-derived" products are marked by vertical lines to the left of the panels. Contaminating activity or activities present in the DEAE fraction and responsible for the labeling of high-molecular-weight products (indicated by brackets to the left of the panels) can be removed by the Myc affinity or the nickel affinity chromatographic procedures. (B) Immunoblotting was used to estimate the degree of Est2p enrichment over the DEAE column. Protein A-tagged Est2p from extracts or DEAE fractions was detected using anti-protein A antibodies. The amount of protein loaded is indicated at the top. The location of the protein A-tagged Est2p is indicated by an arrow. (C) Protein compositions of fractions from successive column steps were analyzed by SDS-PAGE and silver staining. The identities and the amounts of the fractions utilized were as follows: lane 1, extract, 30 μg; lane 2, DEAE, 10 μg; lane 3, phenyl, 3 μg; lane 3, heparin, 1.2 μg; lane 4, IgG, ~0.12 μg. (D) Polymerization assays were carried out using OXYT1 (2 μg) as the DNA primer and fractions from successive column steps. The identities of the fractions used in each reaction were as follows: lane 1, DEAE, 10 μg; lane 2, phenyl, 3 μg; lane 3, heparin, 1.2 μg; lane 4, IgG, ~0.12 μg. The ratios of cleavage to extension products are listed at the top.

OXYT1, the cleavage-derived products were most prominent around the "primer-4" position (Fig. 6B, lanes 1 and 5). This suggests that the telomerase-associated nuclease cleaved DNA preferentially at internal locations, acting as an endonuclease. However, one can also postulate that an exonuclease was responsible and that preferential stalling of the nuclease at particular locations or preferential extension of cleavage products bearing optimal 3′-end sequences gave rise to the observed pattern of product synthesis. To distinguish between these alternatives, we carried out polymerization reactions using primers derivatized with methylphosphonate linkages (Fig. 6A). This modification was expected to render the phosphodiester bond resistant to nuclease attack. If an exonuclease was responsible for the observed primer degradation, the placement of a methylphosphonate linkage between the 3′-most two bases should inhibit the formation of all of the short products. In contrast, if an endonuclease was responsible, then the predominant short products (e.g., the "primer-4" band) should be unaffected because the cleavage that resulted in these products should have occurred far away from the modified linkages.

TABLE 1. Purification of yeast telomerase

Fraction	Protein (mg)	Activity (U)[a]	Fold purification	Cleavage/ extension ratio
Extract	2,100	ND		
DEAE	5.5	1,000	200[b]	0.052
Phenyl	1.8	1,010	620	0.031
Heparin	0.19	270	1,560	0.051
IgG	~0.010	140	~15,400	0.026

[a] Telomerase activity was determined in primer extension assays. Incorporation of labeled dGMP into RNase-sensitive bands was quantified using a PhosphorImager. The total activity of the DEAE fraction was arbitrarily set to 1,000, and the activities of the other fractions were calculated accordingly. ND, not determined.

[b] The fold of purification for the DEAE fraction is based on the degree of Est2p enrichment as determined by Western analysis (Fig. 3B).

As shown in Fig. 6B, some of the cleavage-derived products were retained despite the substitution of one of the two 3′-most phosphodiester linkages of the HS2 or the OXYT1 primer (the HS2-MP1, HS2-MP2, OXYT1-MP1, and OXYT1-MP2 oligonucleotides). These observations suggest that some of the short products must be generated by an endonuclease, as in the case of the *Euplotes* telomerase-mediated cleavage. Close inspection of the reaction products derived from modified HS2 oligonucleotides revealed two interesting features. First, the amount of "direct extension" products (as evidenced by the intensity of the "primer + 3" band) was greatly inhibited by a methylphosphonate at the 3′-most linkage (Fig. 6B, compare lane 2 with lane 1). This suggests that the last phosphodiester linkage of the primer may make a functionally important interaction with telomerase, which can be disrupted by the modification. Methylphosphonate linkages positioned near the 3′ end has also been found to inhibit extension by *E. crassus* telomerase (D. E. Shippen, personal communication). Second, the "primer-4" and "primer-5" products were almost completely abolished (Fig. 6B, compare lane 2 with lane 1), just like the direct elongation products. This similarity suggests that the primer-4 and primer-5 products may also be derived from primers with methylphosphonate modification near the 3′ end. In other words, these products may be due to extension of the 3′ cleavage products. In contrast, the "primer-3" product was virtually unaffected, suggesting that it may be derived from the 5′ fragment generated by the nuclease.

Extensive characterization of primer utilization by yeast telomerase indicates that the enzyme preferentially extends oligonucleotides with 3 Gs at their 3′ end. Furthermore, in our reaction condition, the enzyme has a strong tendency to pause or dissociate after adding 3 nt (TGT) (21; Xia and Lue, unpublished). Taking this property of telomerase into consideration, we can account for all of the cleavage-elongation products of HS2 by the hypothetical scheme presented in Fig. 6C. In this model, cleavage occurs preferentially in the middle G tract. Cleavage between the ninth and tenth nucleotides of HS2 (reaction a) leads to the creation of a 5′ fragment (GG GTTAGGG) that is expected to be a good substrate for telomerase and to yield a predominant 12-nt product (GGGTT AGGGTGT) at the primer-3 location, precisely as was observed. The same cleavage should also give rise to a 3′ fragment (TTAGGG) that can yield a 9-nt product (TTAGG GTGT) at the primer-6 position. This was also observed. Cleavage between the eighth and ninth nucleotides (reaction b) and between the seventh and eighth nucleotides (reaction c), on the other hand, would yield 5′ fragments that are poor substrate for telomerase but 3′ fragments that are good substrates (GTTAGGG and GGTTAGGG). These 3′ fragments are expected to give rise predominantly to the primer-5 and primer-4 products, respectively. An important prediction of this scheme is that the extension products of the 3′ fragments (primer-4, primer-5, and primer-6) should be inhibited by the MP1 modification, while the extension products of the 5′ fragment (primer-3) should not. This prediction was entirely consistent with the observation made here (Fig. 6B, compare lanes 2 and 1). A similar argument can be made to account for the

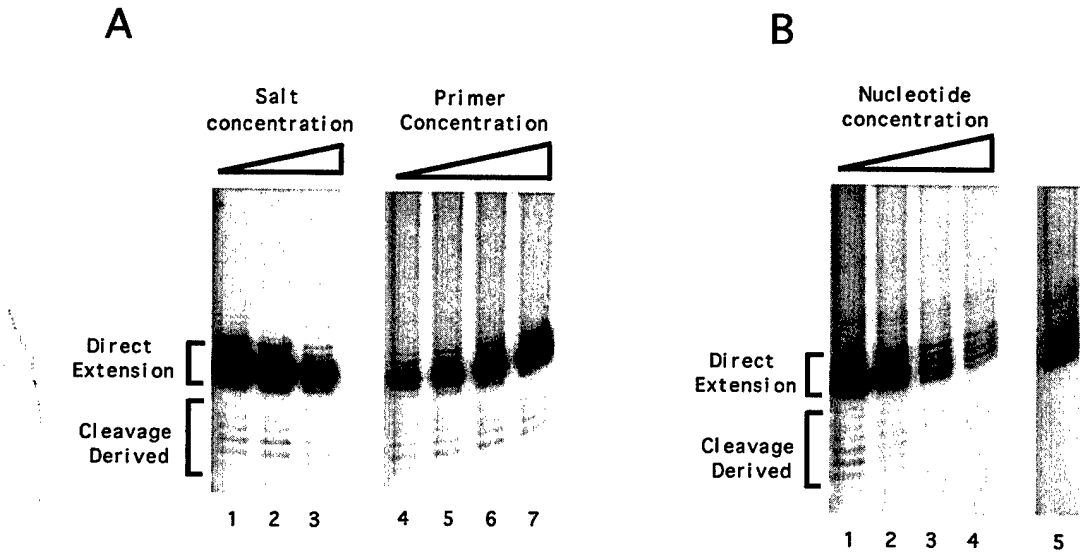

FIG. 4. Effects of salt, primer, and nucleotide concentration on the coupled cleavage and extension reactions mediated by yeast telomerase. (A) Polymerization assays were carried out using OXYT1 as the DNA primer and DEAE column fractions as the source of telomerase. For reactions 1 to 3, the DEAE fraction was first desalted using Centricon-30. Sodium acetate was then added to the following final concentrations: lane 1, 0 mM; lane 2, 150 mM; lane 3, 300 mM. For reactions 4 to 7, the following concentrations of OXYT1 primer were used: lane 4, 3 μM; lane 5, 6 μM; lane 6, 12 μM; lane 7, 24 μM. (B) Polymerization assays were carried out using OXYT1 as the DNA primer and DEAE column fractions as source of telomerase. In addition to 0.2 μM labeled dGTP (3,000 Ci/mmol; NEN), unlabeled dGTP was added to the following concentrations: lane 1, 0 μM; lane 2, 0.5 μM; lane 3, 1.0 μM; lanes 4 and 5, 2.0 μM. Lane 5 represents a longer exposure than lane 4.

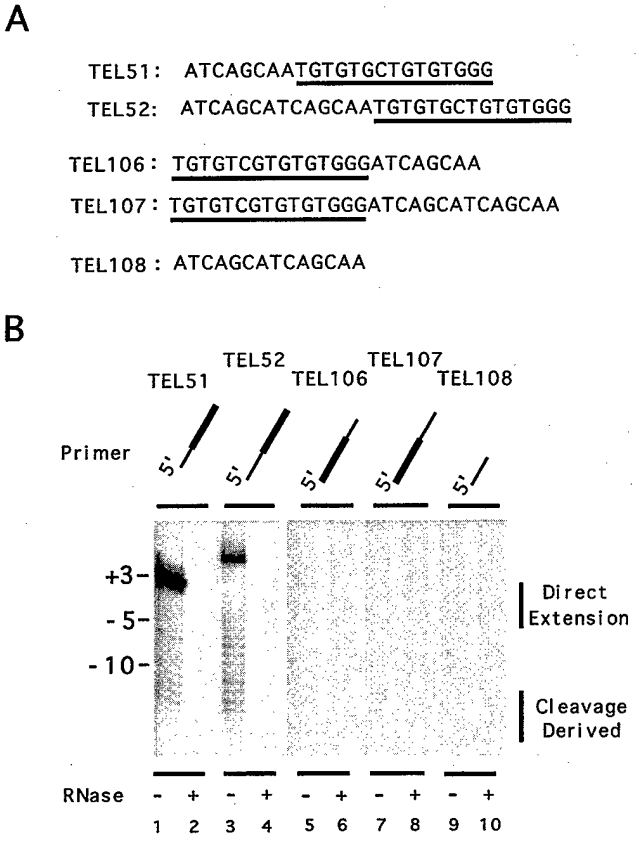

FIG. 5. Effects of flanking nontelomeric cassettes in the DNA primer on cleavage and extension by yeast telomerase. (A) The sequences of the oligonucleotides used for the assays in panel B. The telomeric portion of the primer is underlined. (B) Polymerization assays were carried out using DEAE column fractions as a source of telomerase and various primers as indicated at the top of the panel. The reactions were carried out in the absence or presence of RNase A as indicated at the bottom of the panel. The sizes of the various products in relation to TEL51 (as determined in a separate assay) are indicated to the left, while the regions of the gel containing the direct-extension and cleavage-derived products are indicated by vertical lines to the right of the panel.

cleavage-elongation products of the OXYT1 primer if cleavages occur predominantly in the middle G tract.

Flexibility of the nuclease cleavage site. To test if the predicted cleavage sites were in fact utilized by yeast telomerase, we designed primers (HS2-MP3 and OXYT1-MP3) to specifically render one of the linkages nonhydrolyzable. Both MP3 modifications abolished some but not all of the reaction products, as expected (Fig. 6B, compare lanes 1 and 4 and lanes 5 and 8). More interestingly, the OXYT1-MP3 oligonucleotide gave rise to some products that are not observed in the case of OXYT1. Thus, for OXYT1-MP3, the primer-2 and primer-3 bands are stronger than the primer-4 band, a finding that was precisely the reverse of the pattern for OXYT1 (lanes 5 and 8). These results suggest the interesting possibility that when a preferred cleavage site is resistant to the nuclease, other sites can be utilized, leading to a different distribution of fragments.

Extension of either the 5′ or the 3′ cleavage products by yeast telomerase. Both the nontelomeric cassette study (Fig. 5) and the methylphosphonate substitution study (Fig. 6) suggest that either the 5′ or the 3′ fragment generated by the nuclease can be extended by the RT activity of telomerase. To confirm this conjecture, DNA primers terminating in dideoxynucleotides were synthesized by using terminal transferase and the appropriate dideoxynucleotide triphosphates and then subjected to the extension assay. All products resulting from the addition of nucleotides to the 3′ cleavage fragment were expected to be abolished by this modification, while those from the addition of nucleotides to the 5′ cleavage fragment should be unaffected.

Primers containing nontelomeric cassettes (Fig. 5), as well as primers containing heterologous repeats (Fig. 1, 4, and 6), were tested in this assay. As shown Fig. 7A, when primers bearing 5′ nontelomeric and 3′ telomeric cassettes were utilized, all of the direct extension products can be abolished by substituting the last nucleotide of the primer with dideoxynucleotide, as expected. More significantly, the cleavage-derived products can also be entirely abolished by substituting the last nucleotide of the primer with dideoxynucleotide. However, when the same analysis was applied to primers bearing heterologous telomeric repeats, different results were obtained. For example, substitution of the last dG residue of OXYT1 with ddG eliminated some but not all of the cleavage-derived products (Fig. 7B, lanes 1 and 3). Substitution of the last dG residue of HS2 with ddG had similar effects (Fig. 7B, lanes 5 and 7). Most importantly, in the case of OXYT1 and HS2 the cleavage products eliminated by the ddGMP modification are precisely those eliminated by the MP1 modification, a finding consistent with the notion that both modifications abolished labeling of the 3′ cleavage fragment. For example, the OXYT1-ddG oligonucleotide yielded the prominent primer-4 product (indicated by a closed circle in lane 3) but not the primer-5 or primer-6 products evident in the case of the OXYT1 oligonucleotide (indicated by open circles in lane 3). This was precisely what was observed for the OXYT1-MP1 oligonucleotide. The same comparison can be made between the products generated by HS2-ddG and HS2-MP1 oligonucleotides (compare lane 7 of Fig. 7B and lane 2 of Fig. 6B). As expected for telomerase-mediated extension, all of the cleavage-derived products from either the native or the ddG-modified primers were sensitive to RNase A pretreatment (lanes 2, 4, 6, and 8). Taken together, the dideoxy substitution experiments suggest that in the case of primers containing nontelomeric cassettes, telomerase appears to preferentially extend the 3′ cleavage fragment, while in the case of primers containing heterologous repeats, telomerase appears to be capable of extending both the 5′ and the 3′ fragments derived from cleavage.

DISCUSSION

We have shown that, like ciliate telomerases, yeast telomerase has a tightly associated endonuclease activity that can cleave the starting primer prior to extension by the RT subunit. Novel aspects of this work include the demonstration (i) that the nuclease can be affinity purified along with the RT subunit of telomerase, (ii) that both the 5′ and the 3′ fragments derived from cleavage can be extended by telomerase, and (iii) that the loss of one nuclease site can lead to the preferential utilization of other sites.

The ability of yeast telomerase to extend either one of the cleaved fragments is somewhat surprising in light of earlier studies showing that extension occurs mostly on the 5′ fragment (23). This discrepancy is most likely explained by the use of primers bearing 3′ nontelomeric cassettes in these earlier studies. Such nontelomeric cassettes, once released from the rest of the primers, are probably inefficient substrates for telomerase extension. Indeed, for primers that bear a 5′ nontelomeric cassette and a 3′ telomeric cassette, the cleavage-derived products are all due to labeling of the 3′ fragments, a result consistent with the 3′ telomeric cassettes being better

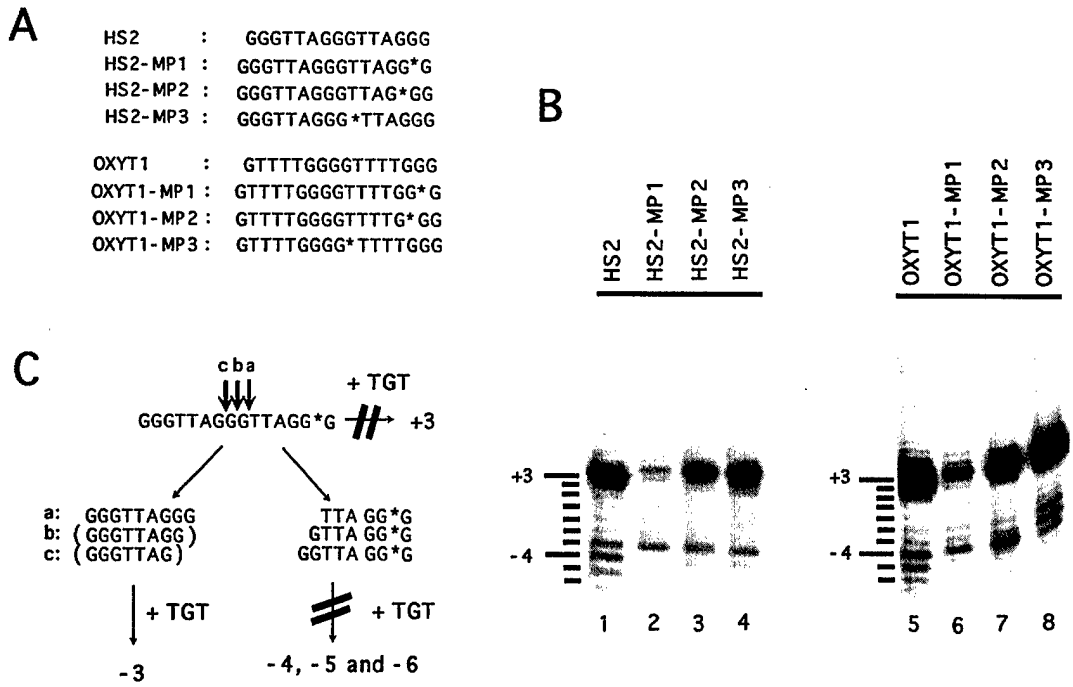

FIG. 6. Effects of methylphosphonate linkages in the DNA primer on cleavage and extension by yeast telomerase. (A) The regular and derivatized oligonucleotides used for the reactions in panel B are listed. The location of the methylphosphonate linkage is denoted by an asterisk. (B) Polymerization assays were carried out using 0.5 μg of the various primers as indicated at the top and either 5 μg (lanes 1 to 4) or 3 μg (lanes 5 to 8) of the DEAE fraction. The lengths of the labeled products relative to the starting primers are indicated by lines and numbers to the left of the panels. (C) A schematic illustration of the cleavage-elongation pathways that can account for the reaction products visualized in lanes 1 and 2 of panel B. The nuclease is proposed to act endonucleolytically and to act predominantly in the middle G tract. As described in the text, yeast telomerase strongly prefers to extend primers that have three Gs at their 3′ end and extends these primers predominantly by 3 nt. Thus, reaction a generates two fragments that can both be efficiently extended, leading to the synthesis of the primer-3 and primer-6 products. Reactions b and c each generate only one efficient substrate, leading to the synthesis of the primer-5 and primer-4 products. The methylphosphonate substitution in MP-1 (marked by an asterisk) strongly inhibits extension of the nearby 3′ OH group by telomerase, causing the loss of the "+3" product as well as the "−4," "−5," and "−6" products.

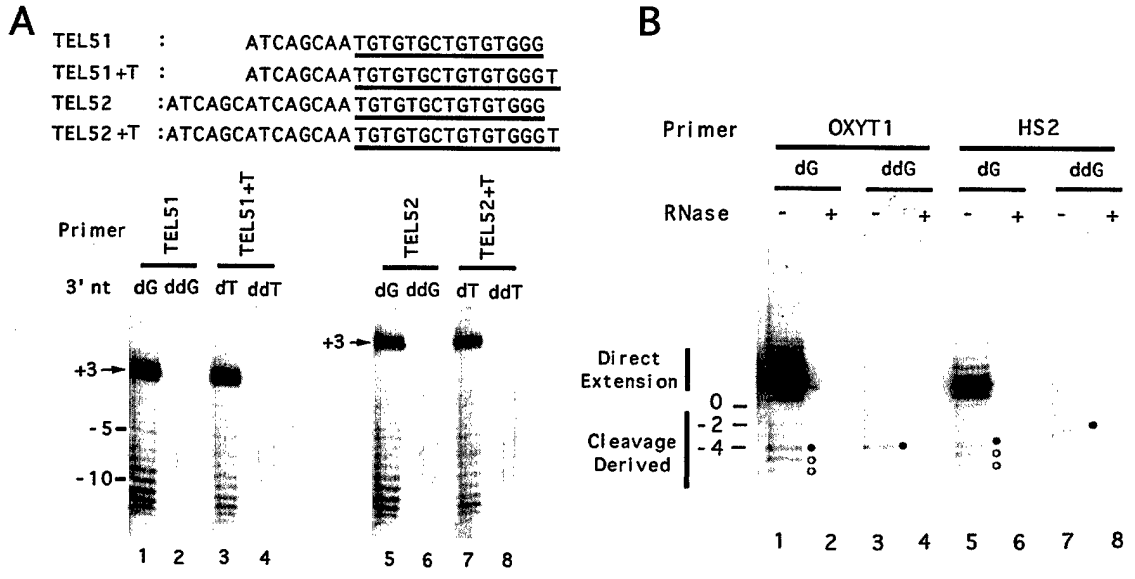

FIG. 7. Effects of 3′ dideoxynucleotide substitutions in the DNA primer on cleavage and extension by yeast telomerase. (A) Polymerization reactions were carried out using 160 ng (lanes 1 to 4) or 400 ng (lanes 5 to 8) of the DNA primers (as indicated at the top of the panels) and 5 μg of the DEAE fractions. The primers bear either a deoxy- or a dideoxynucleotide at their 3′ termini. The sequences of the oligonucleotides used are shown at the top, and the GT-rich (yeast telomere-like) parts of the oligonucleotides are underlined. (B) Polymerization reactions were carried out using 0.5 μg of the DNA primers (as indicated at the top of the panel) and 5 μg of the DEAE fractions. The primers used in lane 3, 4, 7, and 8 bear dideoxynucleotides at their 3′ ends. RNase A was added to the reactions in lanes 2, 4, 6, and 8. Products derived from direct extension or cleavage followed by extension (cleavage derived) are indicated by vertical bars to the left of the panels. Bands unaffected or abolished by the dideoxynucleotide substitution are indicated by closed or open circles, respectively.

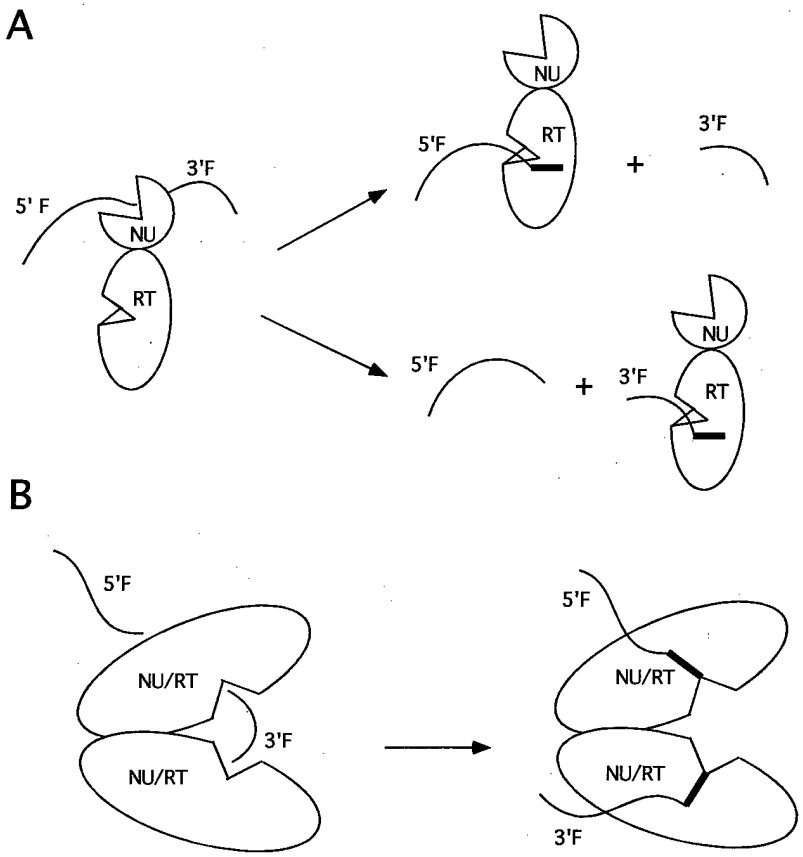

FIG. 8. Models for telomerase-mediated cleavage-extension reactions. (A) Telomerase is shown to possess two distinctive active sites for nuclease (NU) and RT activity. Following cleavage, the RT domain can capture stochastically either the 5′-end fragment (5′F) or the 3′-end fragment (3′F) for extension. (B) Telomerase is shown to be a dimeric enzyme containing two active sites. Each active site is bifunctional and capable of mediating both primer cleavage and extension (NU/RT). Following cleavage of the starting primer by one of the protomers, the resulting 5′-end fragment (5′F) and 3′-end fragment (3′F) can both be extended because of the presence of the two bifunctional active sites.

substrates for yeast telomerase than the 5′ nontelomeric cassettes. Similarly, the ability of yeast telomerase to extend both of the fragments derived from cleavage of heterologous repeats is explained by both fragments' ability to form a hybrid with the RNA template and serve as a substrate for extension.

Initial studies of the *Tetrahymena* enzyme revealed similarities between RNA polymerase-mediated transcript cleavage and telomerase-mediated primer cleavage (5, 36). Such observations raise the interesting possibility that telomerase uses the polymerization site to carry out the cleavage reaction. (The evidence that RNA polymerase mediates transcript cleavage through the polymerization active site is compelling [32]). In this model, one would expect telomerase to extend exclusively the 5′ fragment, because the 3′-OH group of this fragment would be located optimally at the polymerase active site immediately following cleavage. This expectation is clearly not met by our results.

To account for the ability of yeast telomerase to extend both the 5′ and the 3′ fragments generated by cleavage, we propose two general models. The first model postulates two distinct active sites for the RT and nuclease activity in a single polypeptide. These two active sites are flexibly positioned relative to each other such that following the cleavage reaction, the RT domain can stochastically interact with and extend either the 5′- or the 3′-end fragment (Fig. 8A). Existing biochemical data suggest that the telomerase complex can interact with an extended region of the DNA primer, from the 3′ end where polymerization takes place, to approximately 25 nt upstream. Thus, both the 5′ and the 3′ cleavage products may remain associated with the complex and serve as substrates for extension. This general model is consistent with an earlier study by Greene et al. (10) showing a flexible relationship between the nuclease and RT of telomerase. An implication of this model is that a single complex cannot extend both cleavage products simultaneously.

A second plausible model invokes a single active site that mediates both cleavage and extension but postulates that yeast telomerase is multimeric (Fig. 8B). If, for example, telomerase is a dimer, then one protomer can be acting as a nuclease. Following cleavage, this protomer would be ideally positioned to extend the 5′ fragment, while the other protomer can capture the 3′ fragment for extension. In this fashion, a single telomerase complex would be capable of elongating both cleavage products. Consistent with this second model are recently published experimental results showing that yeast telomerase may indeed be multimeric (30). Our two general models are not mutually exclusive, and features of both may be combined. For example, a multimeric telomerase containing distinct nuclease and RT active sites would also be consistent with our experimental results. Clearly, more analysis is necessary to determine the molecular coupling mechanisms between the nuclease and RT of telomerase.

The function of telomerase-associated nuclease remains to be elucidated. That a nonciliate telomerase can be shown to possess a tightly associated nuclease activity indicates that the latter is not likely to be exclusively involved in developmentally mediated chromosome fragmentation. Otherwise, our data are compatible with previously proposed functions, such as enhancing the fidelity of DNA synthesis and enhancing elongation efficiency. Another speculative function for the nuclease is raised by our finding that telomerase may be engaged with the 3′ fragment following cleavage (Fig. 8A). Cleavage in this case can result in the release of the enzyme from telomeric ends and completely abort the elongation of chromosomes. This may be one way of negatively regulating the action of the enzyme. (Extension of the released 3′ fragment would not appear to have any physiologic significance and may simply be an unintended consequence of the cleavage reaction.) Continued analysis of the telomerase-associated nuclease in a genetically tractable organism may eventually allow these proposed functions to be tested in vivo.

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Identification of Functionally Important Domains in the N-Terminal Region of Telomerase Reverse Transcriptase

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Telomerase is a ribonucleoprotein reverse transcriptase responsible for the maintenance of one strand of telomere terminal repeats. The key protein subunit of the telomerase complex, known as TERT, possesses reverse transcriptase-like motifs that presumably mediate catalysis. These motifs are located in the C-terminal region of the polypeptide. Hidden Markov model-based sequence analysis revealed in the N-terminal region of all TERTs the presence of four conserved motifs, named GQ, CP, QFP, and T. Point mutation analysis of conserved residues confirmed the functional importance of the GQ motif. In addition, the distinct phenotypes of the GQ mutants suggest that this motif may play at least two distinct functions in telomere maintenance. Deletion analysis indicates that even the most N-terminal nonconserved region of yeast TERT (N region) is required for telomerase function. This N region exhibits a nonspecific nucleic acid binding activity that probably reflects an important physiologic function. Expression studies of various portions of the yeast TERT in *Escherichia coli* suggest that the N region and the GQ motif together may constitute a stable domain. We propose that all TERTs may have a bipartite organization, with an N-GQ domain connected to the other motifs through a flexible linker.

Telomerase is a ribonucleoprotein (RNP) that is responsible for maintaining the terminal repeats of telomeres in most organisms (15). It acts as an unusual reverse transcriptase (RT), using a small segment of an integral RNA component as template for the synthesis of the dGT-rich strand of telomeres (16).

Telomerase activity has been characterized from a wide range of organisms and genes encoding both the RNA and protein components of the enzyme complex identified (for reviews, see references 2 and 41). Telomerase RNAs found in ciliated protozoa, in addition to having a short templating region, share a common secondary structure. Telomerase RNAs from yeasts and mammals are considerably larger and exhibit no evident structural conservation. The catalytic RT protein subunit (TERT), initially purified from *Euplotes aediculatus* as p123, was subsequently found to be homologous to Est2p, a yeast protein required for telomere maintenance (25, 26, 28). Both proteins possess RT-like motifs, alterations in which led to inactivation of telomerase activity and reduced telomere length. Subsequently, homologs of TERT were identified in *Schizosaccharomyces pombe*, humans, mice, and *Tetrahymena*, *Oxytricha*, and *Arabidopsis* spp. (3, 6, 10, 13, 22, 33, 38, 42). An evident homolog can also be discerned in the incomplete *Candida albicans* database (see Materials and Methods). Additional mutational analysis of the RT motifs in these latter proteins supports a role for TERT in directly mediating catalysis (20, 50). Because coexpression of TERT and telomerase RNA in vitro in the rabbit reticulocyte lysate system suffices to reconstitute enzyme activity (1, 50), these two subunits probably constitute the core of the enzyme complex.

Several groups of telomerase-associated polypeptides have been identified using either biochemical or genetic tools. First, purification of the *Tetrahymena* telomerase complex led to the discovery of two associated polypeptides (p80 and p95). Cloning and characterization of p80 and p95 suggest that these proteins may interact with telomerase RNA and the DNA primer, respectively (8, 12). Mouse and human homologs of p80 have also been identified and been shown to associate with the respective telomerases (19, 39). Second, a significant fraction of the human telomerase complex is apparently associated with the molecular chaperones Hsp90 and p23, which are also necessary for reconstitution of telomerase in vitro activity in the rabbit reticulocyte lysate system (21). These molecular chaperones are hypothesized to play a role in telomerase biogenesis. Third, two Sm proteins that are necessary for snRNA maturation are also components of the yeast telomerase complex, suggesting a role for these factors in telomerase RNA processing and telomerase complex assembly (47). Finally, genetic analysis of yeast identified two Est proteins (Est1p and Est3p) that act in the same pathway as telomerase RNA and TERT. Subsequent characterizations indicate that Est1p and Est3p are also subunits of the telomerase complex and that Est1p may play a role in the recruitment of telomerase core to telomere ends in vivo (9, 25, 41, 49). The precise biochemical and physiologic functions of the telomerase-associated proteins remain to be elucidated. The stoichiometry of telomerase components in the native complex is unclear. However, recent studies suggest that the yeast complex may contain more than one copy of the RNA component (43).

Previous analysis of all of the cloned TERTs revealed several salient features of their structural organization: (i) all of the RT motifs are located in the C-terminal half of the protein; (ii) a telomerase-specific motif (T motif), located just N terminal to the RT motifs, can be discerned in all TERTs; and (iii) a motif positioned further toward the N terminus (CP motif) appears to be much more highly conserved among all the ciliate telomerases and may perform a function specific to ciliate telomere formation (3). Point mutations in conserved

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residues of the T motif significantly impaired telomerase function in the rabbit reticulocyte lysate system (50). Other N-terminal regions of the TERT polypeptide have not been subjected to detailed molecular analysis.

To determine if the uncharacterized, N-terminal regions of TERT are functionally important and if they played conserved roles in telomere maintenance, we initiated mutagenic analysis of the yeast TERT (Est2p). As a starting point for this analysis, we performed a hidden Markov model (HMM)-based alignment of all available TERTs, including the recently identified *C. albicans* homolog. Several earlier applications of the HMM approach have resulted in the detection of homologies between distantly related proteins (34, 35, 37). Interestingly, such an approach in the case of TERT led to the identification of four conserved motifs in the N-terminal, non-RT region. To validate the predictions arising from comparative sequence analysis, we introduced substitution mutations into the yeast TERT (*EST2*) gene and investigated their effects on growth, telomere maintenance, and telomerase activity. In this paper, we report that the most N-terminal motif, called GQ, is indeed functionally important. Characterization of mutant phenotypes suggests that the GQ motif may play at least two distinct functions. Furthermore, we show that a nonconserved region located N terminal to the GQ motif (which we call the N region) is also functionally important, possibly through interacting with some nucleic acid target in the context of the native RNP.

While this work was in progress, Friedman and Cech (11) reported the identification of essential domains located in the N-terminal region of yeast TERT using a unigenic evolution approach. In this approach, the gene of interest is heavily mutagenized, and functional variants are selected. Essential and dispensable regions of the protein are then identified by statistically analyzing the distribution of missense and silent mutations. The regions identified in both their and our studies are largely concordant, and we comment on the similarities and discrepancies in the Discussion section.

MATERIALS AND METHODS

Yeast strains and plasmids. The haploid *Saccharomyces cerevisiae* strain W303-a (MATa *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100*) was used for the construction of the Δ*est2* strain. The disruption cassette was made by inserting ~600 to 800 bp of the *est2* gene flanking sequence upstream and downstream of the kanamycin marker of pUG6. The resulting fragment replaced amino acids 50 to 690 of Est2p open reading frame with the kanamycin resistance marker. Following transformation and selection on G418-containing plates, deletion of the *est2* gene in selected isolates was confirmed by PCR.

The plasmid-borne *est2* gene was derived from yeast strain JX-MH19 and contained at its C terminus both a three-Myc tag and a six-His tag. The JX-MH19 strain was constructed using the PCR recombination method described by Schneider et al. (46). The pMPY-3XMYC plasmid was amplified using the following two primers: EST2TAG1, 5′-AAGATAATATCATTCTTTTGA GAAAGGAAATTCAACACTTGCAAGCAAGGGAACAAAAGCTGG; and EST2TAG2, 5′-CCTTATCAGCATCATAAGCTGTCAGTATTTCATGTAT TATTAGTACTACTAGTGATGGTGATGGTGATGTAGGGCGAATTGGG TACC. The disruption cassette was introduced into W303-a, and transformants were selected on a Ura⁻ plate. Subsequent selection in the presence of 5-fluoroorotic acid allowed homologous recombination of the repeated Myc tags such that the chromosomal copy of the *EST2* gene became fused at its C terminus with a three-Myc tag and a six-His tag. Telomere lengths and telomerase activity in this strain (JX-MH19) are comparable to those of W303-a. To introduce this modified *EST2* gene onto a plasmid, a 3.6-kb fragment encompassing the modified *est2* coding region (and containing ~500 bp of upstream region and ~500 bp of downstream region) was amplified by PCR and inserted between the *Bam*HI and *Sal*I sites of pSE358 to give pSE-Est2TA. To allow for the construction of deletion mutants, the sequence surrounding the start codon of pSE-Est2TA was converted to an *Nde*I site by site-directed mutagenesis to give pSE-Est2Nde. Additionally, a protein A-tagged *est2*-containing plasmid was constructed by inserting two copies of the immunoglobulin G (IgG) binding domain of protein A (generated by PCR from pEZZ 18) between the *Xho*I and *Kpn*I sites of pSE-Est2Nde to give pSE-Est2-proA.

For deletion mutants, fragments encompassing amino acids 10 to 119, 20 to 119, 30 to 119, and 50 to 119 of Est2 were amplified by PCR and inserted between the *Nde*I and *pfl*MI sites of pSE-Est2Nde to generate the desired plasmids. All point mutations were generated by using the Quick-Change protocol (Stratagene), appropriate primer oligonucleotides, and pSE-Est2Nde as template. All point mutations were confirmed by sequencing. Some of the deletion and point mutants were also made with the protein A tag by transferring the *Nde*I-to-*Nco*I fragment from the mutated pSE-Est2Nde plasmid to the pSE-Est2-proA plasmid.

To overexpress Est2p, a vector containing the triose phosphate isomerase promoter (pYX212 from Ingenious Inc.) was utilized. The *Nco*I site within the polylinker of pYX212 was converted to an *Nde*I site, and the *Nde*I-*Sal*I fragments from the pSE-Est2Nde series of plasmids (containing wild-type or mutated EST2) were inserted between the *Nde*I and *Sal*I sites of the resulting vector.

Sequence comparison. All sequences used in comparative analysis, with the exception of the *C. albicans* TERT homolog, were obtained from GenBank. Sequence data for *C. albicans* was obtained from the Stanford DNA Sequencing and Technology Center website at http://www-sequence.stanford.edu/group/candida.

Determination of telomere length. Chromosomal DNA was isolated using the Smash and Grab protocol, digested with *Xho*I or *Pst*I restriction enzyme, and electrophoretically separated on a 1% agarose gel. Following capillary transfer to nylon membranes, telomere-containing fragments were detected by hybridization with a ^{32}P-labeled poly(dG-dT) probe.

Purification of and assay for yeast telomerase. Whole-cell extracts and active DEAE fractions were prepared as previously described (5, 29, 30). 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 dithiothreitol, 5% glycerol (contributed by the protein fraction), 50 μM dTTP, 20 μCi of [α-^{32}P]dGTP (3,000 Ci/mmol), 5 μM primer oligodeoxynucleotides (TEL66 [30]), and 15 μl of column fractions. Primer extension products were processed and analyzed by gel electrophoresis as previously described (29, 30). Signals were quantified using a PhosphorImager system (Molecular Dynamics). For quantification of activity, the signals from all labeled and RNase-sensitive products are summed.

For affinity depletion of protein A-tagged yeast telomerase, about 100 μl of DEAE fraction (in a buffer that contains ~700 mM sodium acetate) was directly incubated with 5 μl of IgG-Sepharose beads (Pharmacia) at 4°C with gentle rocking for 2 h. The beads were pelleted by centrifugation, and the supernatant was assayed for activity.

Western analysis of protein A-tagged Est2p. Depending on the expression level, the amount of protein A-tagged Est2p was analyzed either directly in crude extracts or following IgG-Sepharose precipitation using the ProtoBlot system (Promega). For analysis using crude extracts, ~300 to 500 μg of total protein was electrophoresed into a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel and transferred to nitrocellulose membrane. Primary anti-protein A antibody (Sigma) and secondary antibody were used at 1:1,000,000 and 1:5,000 dilutions, respectively. The high primary antibody dilution was necessary to minimize background arising from cross-reacting polypeptides. For analysis using IgG-Sepharose-purified telomerase, ~5 mg of unfractionated extracts was treated with 40 μl of IgG-Sepharose at 4°C for 16 h. The beads were washed three times with TMG-10(600), and bound proteins were eluted with SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer. Following electrophoresis and blotting, Est2p was detected by using primary and secondary antibody at 1:20,000 and 1:5,000 dilutions, respectively.

Construction of *Escherichia coli* EST2 fusion protein expression plasmids and purification of fusion proteins from *E. coli*. Fragments of the *EST2* gene were amplified by PCR and cloned between the *Bam*HI and *Pst*I sites of the pMAL-cri vector (New England Biolabs). In addition, the downstream primers all possessed sequences that encode an in-frame six-His tag, allowing the fusion proteins to be purified using both nickel-affinity and amylose-affinity chromatography. The proteins expressed from these plasmids are named MBP-Est2(1–160)p, etc., with the numbers in parentheses indicating the amino acid residues of Est2p included in the fusion protein.

The plasmids carrying the maltose-binding protein (MBP)–*EST2* fusion genes were transformed into BL21 cells. Transformants were inoculated into Luria-Bertani broth and grown under ampicillin selection (50 μg/ml) at 37°C overnight. A 10-ml culture of saturated cells was diluted with 1 liter of Luria-Bertani broth with ampicillin and grown at 37°C for 2.5 h. When the optical density at 600 nm of the culture reached 0.3 to 0.5, the cultures were cooled down to room temperature and induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to 1 mM and growth at room temperature for 3 additional h. The cells were harvested, and extracts were prepared by sonication. Fusion proteins were purified successively over an Ni-nitrilotriacetic acid resin (Qiagen) and an amylose resin (Bio-Lab) according to the manufacturers' instructions. All preparations were found to be >90% pure by SDS-PAGE and Coomassie blue staining.

Filter binding assay. Protein-nucleic acid binding assays were performed using a modification of a published procedure (4). Est2p fusion proteins (from 0.25 to 5 μg) were mixed with ^{32}P-labeled nucleic acid and 50 μg of bovine serum albumin in 10 mM Tris (pH 8.0) and 10% glycerol. The reaction mixtures (50-μl total volume) were incubated on ice for 30 min and pipetted over a prewetted nitrocellulose filter (BA85; Schleicher & Schuell) sandwiched in a slot blot apparatus. The reaction mixtures were then slowly filtered through the mem-

brane using gentle suction. The filters were washed three times with 0.5 ml of buffer (10 mM Tris, 300 mM sodium acetate, 50 μg of bovine serum albumin), air dried, and exposed to a PhosphorImager screen (Molecular Dynamics). All assays were done in duplicate, and the signals were averaged for further analysis. The difference between duplicate samples was generally <10%.

RESULTS

HMM-based analysis revealed four conserved motifs located in the N-terminal, non-RT region of TERT. To determine if the N-terminal, non-RT regions of TERT are functionally important and if they play conserved roles in telomere maintenance, we initiated mutagenic analysis of Est2p (the yeast TERT). Several earlier applications of HMM-based sequence comparison resulted in detection of homologies between distantly related proteins (34, 35, 37). Therefore, as a starting point for our analysis, we performed an HMM-based alignment of all available TERTs, including recently identified *Arabidopsis thaliana* and *C. albicans* homologs. After obtaining an alignment of the entire amino-terminal region, we defined the motif-domain boundaries by visual inspection. The criteria used for assigning regions of the alignment as linkers included low compositional complexity, absence of clusters of conserved residues, and large numbers of gaps in the majority of sequences. Interestingly, this protocol identified four conserved motifs in the N-terminal, non-RT region of TERT (Fig. 1). We call these motifs in order from N to C terminus the GQ, CP, QFP, and T motifs, respectively. The locations of these motifs within Est2p are as follows: GQ, residues 45 to 163; CP, residues 245 to 265; QFP, residues 267 to 343; and T, residues 367 to 413. Each motif contains nearly invariant amino acid residues that are located at fixed distances from one another. The GQ and QFP motifs have not been previously recognized. In addition, the CP motif, hypothesized earlier to be ciliate specific, is shown in our analysis to possess invariant and nearly invariant residues. The identification of conserved motifs throughout the N-terminal region of TERT suggests that this region mediates a conserved function(s) in telomere synthesis. The overall comparison also revealed a particularly degenerate region that is variable in length, located between motifs GQ and CP, implying the presence of a flexible linker (Fig. 1B).

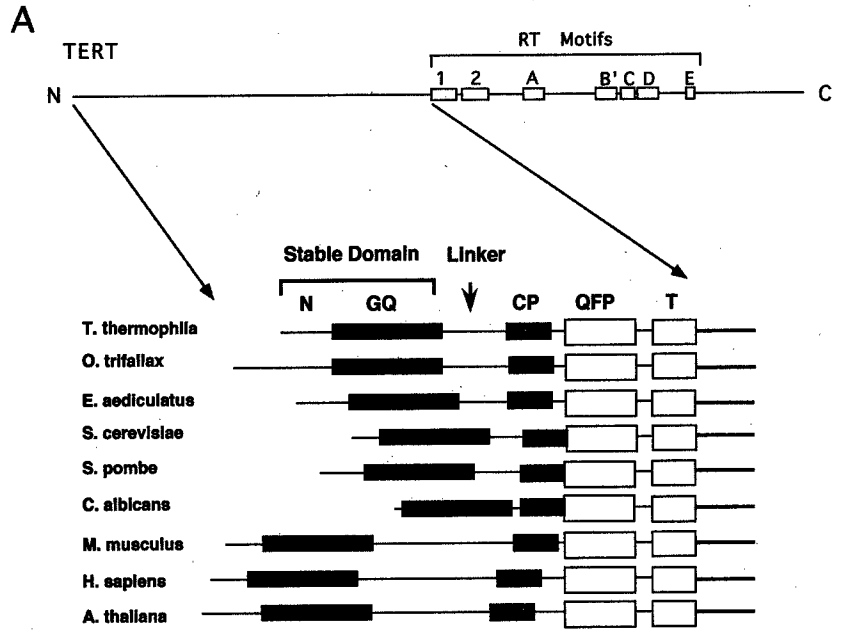
The GQ motif is required for telomere maintenance. To validate some of the predictions arising from comparative sequence analysis, we constructed mutants of the *EST2* gene bearing alanine substitutions in conserved residues in the GQ motif. The mutated *EST2* gene, located on a centromeric shuttle vector under the control of its natural promoter, was transformed into an *est2*::Kan^r strain that had been grown in the absence of Est2p for ~25 to 50 generations. The resulting strain was then monitored for defects in growth, telomere maintenance, and telomerase activity. To facilitate future biochemical analysis, the plasmid-borne *EST2* gene was fused at its 3′ end with three tandem Myc tags and a six-His tag. In some cases, an additional protein A tag (consisting of two copies of the IgG binding domain from protein A) was inserted in between the Myc and His tags to allow even more efficient affinity purification. These C-terminal modifications have no effect on telomere maintenance and telomerase activity (J. Xia, unpublished data).

A total of eight alanine substitutions at conserved GQ motif residues were constructed and tested: D66A, G85A, N104AV105A, W115A, F118AH119A, G123A, Q138AF139A, and G141A. Two additional mutants with substitutions in nonconserved residues (D93A and E154A) were also made and tested for comparison. As summarized in Table 1, three of these mutants (W115A, F118AH119A, and G123A), located quite close to one another, exhibited the most pronounced growth defects, giving rise to small and variably sized colonies suggestive of senescence (31). The senescent phenotype, characterized by progressively slower cell multiplication, has been observed in several other telomerase knockout strains (25, 48). The display of senescence suggests that the W115A, F118AH119A, and G123A mutants are quite compromised in telomerase function. In contrast, the other mutants either did not show any growth defects or grew only slightly slower (compared to a strain carrying a plasmid containing the wild-type *EST2* gene) and failed to exhibit signs of senescence upon repeated restreaking.

Chromosomal DNA was isolated from individual transformants following two restreaks (~50 generations) and assayed for telomere lengths. As shown in Fig. 2, the Y′ class of telomeres in all mutants with substitutions in conserved residues shows dramatic telomere shortening of at least 150 to 200 bp (all mutants except D93A and E154A, lanes 2 to 5, 8 and 9, and 12 to 21). The senescent mutants have even shorter telomeres and gave especially weak signals for hybridization (W115A, F118AH119A, and G123A, lanes 12 to 17). Thus, there is a good correlation between growth defects and telomere repeat loss, consistent with earlier findings. In contrast to mutations in conserved residues, the two strains with mutations in nonconserved residues (D93A and E154A) failed to show any evidence of telomere shortening.

Two mutations impaired telomere maintenance without affecting telomerase activity. Because several studies point to the existence of mutations that uncouple in vitro telomerase activity from in vivo telomere maintenance (e.g., *est1* and *est3* mutants [27]), we were interested in determining if any of the GQ mutations had similar properties. Extracts were prepared from each of the mutant strains, and telomerase activity was partially purified over DEAE columns and tested. Earlier studies indicated that, under standard reaction conditions, this chromatographic fraction yields labeled products that are almost entirely due to TLC1 and Est2p (5; N. Lue, unpublished data). As shown in Fig. 3A, almost the entire primer extension signal in this preparation is sensitive to RNase A pretreatment. In addition, when the Est2p in the strain is tagged with two copies of the IgG binding domain from protein A, >80% of the activity in the DEAE fraction can be specifically trapped on IgG-Sepharose beads and depleted from supernatant, indicating

FIG. 1. Identification of conserved motifs in the N-terminal, non-RT region of telomerase RT. (A) A schematic illustration of the locations of RT motifs in the telomerase RT polypeptide as determined by an earlier analysis is shown at the top (3). HMM-based analysis revealed four conserved motifs located in the N-terminal region of all TERTs that have been identified thus far (from *Tetrahymena thermophila*, *Oxytricha trifallax*, *E. aediculatus*, *S. cerevisiae*, *S. pombe*, *C. albicans*, *Mus musculus*, *Homo sapiens*, and *A. thaliana*). These motifs are named in order from N to C terminus the GQ, CP, QFP, and T motifs, respectively. For ease of discussion, we also designate the most N-terminal nonconserved region of TERT the N region. The segment between the GQ motif and the CP motif is particularly variable in length, consistent with the existence of a flexible linker in this region. (B) A detailed alignment of the four motifs is shown. The GQ motif is shaded yellow, and the CP, QFP, and T motifs are shaded gray. Invariant residues are italicized, and highly conserved residues are shown in red. Conserved hydrophobic residues are shown in boxes. Closed circles denote point mutations described in this study, and open triangles denote functional mutations at nonconserved positions as reported by Friedman and Cech (11). Functional amino acid substitutions at conserved positions are explicitly given at the bottom. The numbers in parentheses are the numbers of functional mutations in connecting regions. Regions I, II, III, and IV as defined by Friedman and Cech (11) are indicated at the top. Sequences are from *T. thermophila*, *C. trifallax*, *E. aediculatus*, *S. cerevisiae*, *S. pombe*, *C. albicans*, *M. musculus*, *H. sapiens*, and *A. thaliana* (top to bottom, respectively).


A
TERT
RT Motifs
1 2 A B' C D E
N
C
Stable Domain
Linker
N GQ CP QFP T
T. thermophila
O. trifallax
E. aediculatus
S. cerevisiae
S. pombe
C. albicans
M. musculus
H. sapiens
A. thaliana

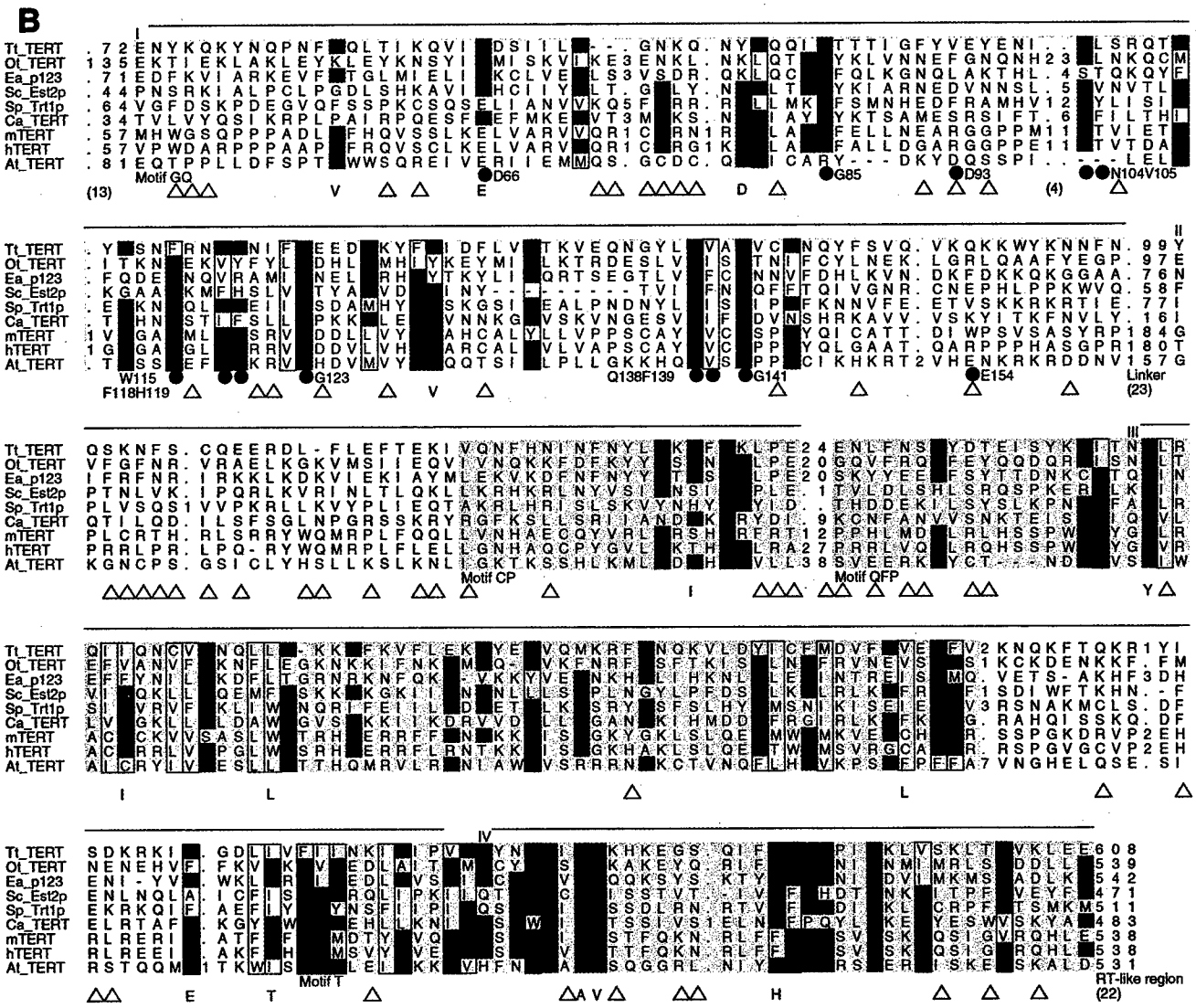

B
Motif GQ
Motif CP
Motif QFP
Motif T
RT-like region
Linker

TABLE 1. Summary of in vivo and in vitro phenotypes of point mutants in the GQ motif

Mutation	Senescence[a]	Telomeres[b]	Primer extension activity[c]
None	−	+++	100
D66A	−	+	70
G85A	−	+	8
D93A	−	+++	140
N104AV105A	−	+	80
W115A	+	+/−	<2
F118AH119A	+	+/−	<2
G123A	+	+/−	<2
Q138AF139A	−	+	5
G141A	−	+	6
E154A	−	+++	100

[a] Senescent strains (+) grew slowly on the initial transformation plate and gave rise to small colonies that are the same size as those from an Δ*est2* strain transformed with the pSE358 vector.

[b] Telomere lengths were determined in Southern hybridization assays (Fig. 2) and were scored as follows: +++, wild-type telomere length; +, on average about 150 to 200 bp shorter; +/−, on average about 300 bp shorter with weak hybridization signals.

[c] Primer extension assays were performed using TEL66 as primer under standard conditions. Each mutant fraction was assayed alongside the wild-type fraction. The signals obtained from the mutant fractions were normalized against that from the wild-type fraction, which was taken as 100.

that this fraction is largely free of other contaminating activities (Fig. 3A).

Each mutant telomerase was tested side by side with the wild-type fraction using equal amounts of total protein (Fig. 3B). As expected, the control mutants that exhibited no telomere shortening had in vitro activities that were comparable to those of the wild-type enzyme (D93A and E154A, lanes 5 and 15). This result further demonstrates the reproducibility of our protocol. Six of the eight mutants with substitutions in conserved residues exhibited greatly reduced telomerase activity, ranging from ~12-fold reduction for G85A (lane 3) to more than 50-fold reduction for the senescent mutants (W115A and G123A, lanes 9 and 10). Interestingly, two mutants, D66A and N104AV105A (lanes 2 and 7), appear to uncouple telomerase activity in vitro from telomere maintenance in vivo; though the telomeres in these two strains are greatly shortened, the mutant enzymes exhibited nearly wild-type levels of activity. Other strains that had similar telomere length defects such as G85 and Q138AF139A (lanes 3 and 12) suffered a ~12- to 20-fold reduction in telomerase activity (Table 1). All mutant fractions were assayed at least twice, and where a significant reduction in activity relative to that of the wild-type fraction was observed, the mutant activity as a percentage of the wild-type activity varied by less than 5%.

The extreme N-terminal region of Est2p (N region) is also required for telomere maintenance in vivo and telomerase activity in vitro. Our comparative analysis suggests that the extreme N-terminal 50 amino acids (termed N region) of yeast TERT could not be reliably aligned with its homologs from other species. To test the importance of this N region, we constructed N-terminal truncation mutants of the *EST2* gene and tested their ability to support telomere length maintenance and telomerase activity using the previously described system.

Four deletion mutants missing amino acids 2 to 10, 2 to 20, 2 to 30, and 2 to 50 (abbreviated as N-10, N-20, N-30, and N-50, respectively) were tested in this system. As shown in Fig. 4A and Table 2, the N-10 and N-20 mutants exhibited significantly reduced growth rates in minimal medium on agar plates. However, they showed no evidence of senescence upon repeated restreaking (data not shown). In contrast, the N-30 and N-50 mutants gave rise to heterogeneously sized colonies suggestive of senescence. Chromosomal DNA from two independent clones of each strain was isolated following two restreaks and analyzed for telomere length by Southern hybridization. Consistent with the growth defects, all deletion mutants, including the smallest one (N-10), possessed significantly shortened telomeres relative to the control strain (Fig. 4B, compare lanes 1 and 8 with lanes 2 to 7). Both Y′-type telomeres (marked by a vertical bar) and X-type telomeres (marked by arrows) in the mutant strains were shortened, consistent with a general defect in telomere maintenance.

The deletion mutants were also tested for defects in telomerase activity using the primer extension assay. As shown in Fig. 4C, fractions derived from the N-10 and N-20 strains exhibited significantly reduced telomerase activity compared to those from the control strain. Quantification by PhosphorImager analysis indicates that the reduction is approximately 40- to 50-fold. The N-30 and N-50 strains were not tested for in vitro activity. However, based on the growth defects, it is likely that these latter strains would have exhibited the same or less telomerase activity. We conclude that the N region of Est2p is required for full telomerase primer extension activity as measured in vitro.

Loss of telomerase activity in the mutants cannot be accounted for by loss of protein expression-stability alone. To determine if reduced telomerase activity of the deletion and point mutants was due to reduced expression-stability of Est2p, we determined the amount of protein A-tagged Est2p in the mutant strains by Western analysis. To minimize potential variations introduced by IgG-Sepharose binding, we first directly analyzed unfractionated extracts for the presence of

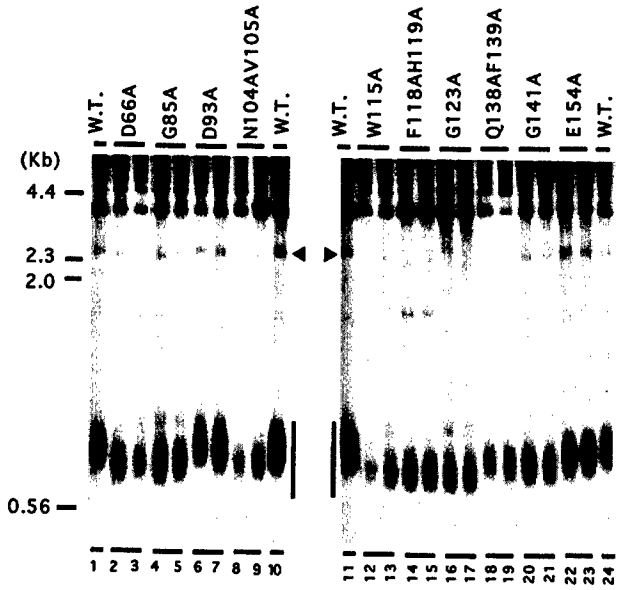


FIG. 2. Telomere length determination in strains that contain mutations in the conserved and nonconserved residues within the GQ motif. The Δ*est2* strain that had been grown for ~25 to 50 generations was transformed with plasmids bearing either wild-type (W.T.) or mutated *EST2*. The transformants were restreaked twice, and single colonies were picked for growth in liquid culture. Chromosomal DNAs were isolated from the cultures, digested with *PstI*, electrophoresed into a 1% agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled poly(dG-dT). The locations of the Y′ telomeres and an X telomere are indicated by a vertical bar and an arrowhead, respectively. The mobilities of several molecular size standards are indicated on the left.

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Functional Analysis of Conserved Residues in the Putative "Finger" Domain of Telomerase Reverse Transcriptase*

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Telomerase is a ribonucleoprotein reverse transcriptase (RT) responsible for the maintenance of one strand of telomere terminal repeats. The catalytic protein subunit of telomerase, known generically as telomerase reverse transcriptase (TERT), exhibits significant homology to RTs encoded by retroviruses and retroelements. The polymerization mechanisms of telomerase may therefore be similar to those of the "conventional" RTs. In this study, we explored the extent of mechanistic conservation by analyzing mutations of conserved residues within the putative "finger" domain of TERT. Previous analysis has implicated this domain of retroviral RTs in nucleotide and RNA binding and in processivity control. Our results demonstrate that residues conserved between TERT and human immunodeficiency virus-1 RT are more likely than TERT-specific residues to be required for enzyme activity. In addition, residues presumed to make direct contact with either the RNA or nucleotide substrate appear to be functionally more important. Furthermore, distinct biochemical defects can be observed for alterations in the putative RNA- and nucleotide-binding TERT residues in a manner that can be rationalized by their postulated mechanisms of action. This study thus supports a high degree of mechanistic conservation between telomerase and retroviral RTs and underscores the roles of distinct aspects of telomerase biochemistry in telomere length maintenance.

Telomerase is a ribonucleoprotein that is responsible for maintaining the terminal repeats of telomeres in most organisms (1). It acts as an unusual reverse transcriptase, using a small segment of an integral RNA component as template for the synthesis of the dGT-rich strand of telomeres (2).

Telomerase activity has been characterized in a wide range of organisms and genes encoding both the RNA and protein components of the enzyme complex identified (for reviews, see Refs. 3 and 4). Telomerase RNAs found in ciliated protozoa, in addition to having a short templating region, share a common secondary structure. Telomerase RNAs from yeast and mammals are considerably larger; and within each group, conserved structural elements can be identified based on phylogenetic and mutational analysis (5, 6). The catalytic reverse transcriptase protein subunit TERT,[1] first purified from *Euplotes aediculatus* as p123, was found to be homologous to Est2p, a yeast protein required for telomere maintenance (7–9). Both proteins possess reverse transcriptase (RT)-like motifs, alterations in which can result in inactivation of telomerase activity and reduced telomere length. Subsequently, homologs of TERT were identified in *Schizosaccharomyces pombe*, human, mouse, *Tetrahymena*, *Oxytricha*, and *Arabidopsis* (10–17). Mutational analysis of the RT motifs in these latter proteins further supports a role for TERT in directly mediating catalysis (18, 19). Because coexpression of TERT and telomerase RNA *in vitro* in the rabbit reticulocyte lysate system suffices to reconstitute enzyme activity (18, 20), these two subunits probably constitute the core of the enzyme complex. Several telomerase-associated polypeptides have been identified using either biochemical or genetic tools. Preliminary studies suggest that these factors may participate in telomerase assembly, function, or regulation (21–25).

As mentioned above, mutational analysis of TERT residues equivalent to those located within functional motifs of conventional RTs supports an overall conservation of basic catalytic mechanisms between these two classes of enzymes. For example, the TERT analogs of RT residues essential for catalysis are absolutely required for telomerase activity and telomere maintenance (9, 18, 19, 26, 27). Conserved residues previously shown to modulate RT processivity have been found to be important determinants of telomerase processivity as well (28, 29). In addition, the same tyrosine residue in conserved motif A allows both TERT and RTs to discriminate against incorporating ribonucleotides (30). However, some other crucial RT residues (*e.g.* Gln in motif B′) appear to be less important or even dispensable in telomerase (9). Together, these results suggest that despite the high degree of sequence divergence (<20% sequence identity), TERT and conventional RTs may possess very similar polymerization mechanisms.

We have sought to clarify the extent of mechanistic conservation between TERT and conventional RTs by comparative analysis of HIV-1 RT and *Saccharomyces cerevisiae* TERT properties. Specifically, we mutagenized *S. cerevisiae* TERT residues that, according to alignment and the structure of the HIV-1 RT-substrate complex (31), are presumed to mediate important aspects of the polymerization reaction. We then subjected the resulting telomerase to detailed biochemical and

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[1] The abbreviations used are: TERT, telomerase reverse transcriptase; RT, reverse transcriptase; HIV-1, human immunodeficiency virus-1.

genetic analysis. Previous studies (29) focused on regions of *S. cerevisiae* TERT equivalent to the "palm" and "thumb" domains of HIV-1 RT and revealed striking similarities between these enzymes with respect to processivity control. Specifically, conserved motifs C and E and a C-terminal extension, all previously demonstrated to be processivity determinants of HIV-1 RT, were found to govern processivity of *S. cerevisiae* TERT as well. In addition, a correlation was demonstrated between telomerase processivity and the equilibrium length of telomeres (29), suggesting a causal relationship between the two parameters. In this study, we undertook a more detailed analysis of the putative "finger" domain of *S. cerevisiae* TERT, presumed to function in *nucleotide* and *template* binding. Residues believed to interact with the nucleotide substrate and the RNA template were mutated, and the resulting telomerase was subjected to biochemical and genetic analysis. Consistent with a high degree of mechanistic similarity, we show that residues conserved between *S. cerevisiae* TERT and HIV-1 RT are more likely than TERT-specific residues to be required for enzyme activity. In addition, altering residues that are presumed to make direct contact with either the RNA or nucleotide substrate caused greater physiologic defects. Clear functional differences can be observed between the RNA- and nucleotide-interacting TERT residues in a manner that can be rationalized by their postulated mechanisms of action. Our observations also underscore the importance of telomerase processivity in controlling telomere length.

MATERIALS AND METHODS

Yeast Strains and Plasmids—The construction of a Δ*est2* strain harboring the pSE-Est2-C874 plasmid (containing a protein A-tagged *EST2* gene) has been described (32). All point mutations in the finger domain of *EST2* were generated using the QuickChange protocol (Stratagene), appropriate primer oligonucleotides, and pSE-Est2-C874 as template. All point mutations were confirmed by sequencing.

Primers—The oligodeoxynucleotide primers used for telomerase assays were purchased from Sigma and were purified by denaturing gel electrophoresis prior to use.

Purification of and Assay for Yeast Telomerase—Whole cell extracts and IgG-Sepharose-purified telomerase were prepared as previously described (29, 32–34). Each primer extension assay was carried out using 20 μl of IgG-Sepharose pretreated with 4 mg of protein extract and was initiated by the addition of a 15-μl mixture containing 100 mM Tris-HCl (pH 8.0), 4 mM magnesium chloride, 2 mM dithiothreitol, 2 mM spermidine, 10 μM primer oligodeoxynucleotides, and varying combinations of labeled and unlabeled dGTP and dTTP. Primer extension products were processed and analyzed by gel electrophoresis as previously described (34, 35). All assays were performed in duplicates or triplicates to allow for determination of averages and deviations.

For determination of processivity, the signal for each product was determined by a PhosphorImager (Molecular Dynamics, Inc.) and normalized to the amount of transcript by dividing by the number of labeled residues. Both the TEL15 and TEL66 primers were designed such that they can align to only one site along the yeast RNA template and support the addition of a specific sequence (TGTGGTG). The processivity for each position (P_i) was calculated using the following formula: $P_i = \text{sum}(T_{i+1} + T_{i+2} + \ldots + T_n)/\text{sum}(T_i + T_{i+1} + \ldots + T_n)$, where T_i designates the amount of transcript calculated for the P+i position, and n designates the highest number such that a visible signal can be discerned in the PhosphorImager file for the P+n product.

RNase Protection Analysis—IgG-Sepharose-enriched telomerase was prepared as described above and deproteinated by SDS and proteinase K treatment and phenol extraction. The remaining nucleic acids were combined with an antisense probe (100,000 cpm) and hybridized and digested as previously described (36). For synthesis of uniformly labeled RNA probe, the *TLC1* gene (nucleotides 1–1301) (37) was first amplified by polymerase chain reaction and cloned in between the *Bam*HI and *Eco*RV sites of pBluescript II KS$^+$. The resulting plasmid was linearized by digestion with *Hin*fI, and antisense RNA encompassing residues 1097–1301 of the *TLC1* gene was generated by T3 RNA polymerase in the presence of 12 μM [α-^{32}P]GTP as described (36).

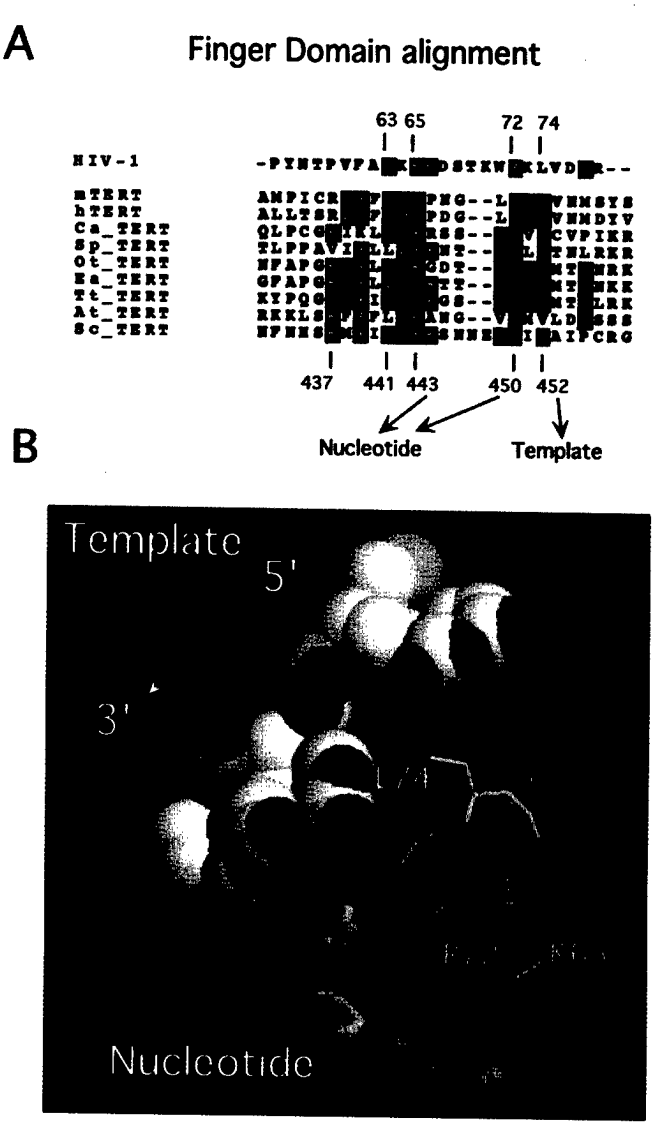

FIG. 1. **Sequence and structure of the finger domain of HIV-1 RT and its alignment with TERTs.** *A*, an alignment of the finger domains of HIV-1 RT and nine different TERTs is shown. Highly conserved residues are displayed in *black boxes*. The residue numbers for mutated *S. cerevisiae* TERT amino acids are indicated below, and the numbers for the corresponding HIV-1 RT amino acids are indicated above. Residues contacting either the nucleotide or the template in the HIV-1 RT structure are also marked. *m*, mouse; *h*, human; *Ca*, *Candida albicans*; *Sp*, *S. pombe*; *Ot*, *O. trifallax*; *Ea*, *E. aediculatus*; *Tt*, *Tetrahymena thermophila*; *At*, *Arabidopsis thaliana*; *Sc*, *S. cerevisiae*. *B*, the spatial locations of the key finger domain amino acid residues in relation to the template and surrounding bases and the nucleotide triphosphate substrate are illustrated. The templating and surrounding bases are shown as space-filling models, whereas the amino acids and nucleotide triphosphate shown as sticks. Leu^{74} of HIV-1 RT is shown with a *dotted surface* to emphasize its packing against the template base. The coordinates are from Huang *et al.* (31).

RESULTS

Point Mutations in the Putative Finger Domain of TERT Can Severely Compromise Telomerase Function in Vivo—To clarify the extent of mechanistic conservation between retroviral RTs and TERT, we mutagenized residues in the finger domain of yeast TERT (Est2p) that are conserved either within the TERT family or within the larger RT family and tested the resulting polypeptides for function both *in vitro* and *in vivo*. In a published comparative sequence analysis of RTs (50), the finger domain comprises RT motifs 1 and 2, located in close proximity and each consisting of ~3–5 highly conserved amino acid residues (Fig. 1*A*). In atomic resolution structural models of HIV-1 RT, motifs 1 and 2 constitute a long β-hairpin, the base of

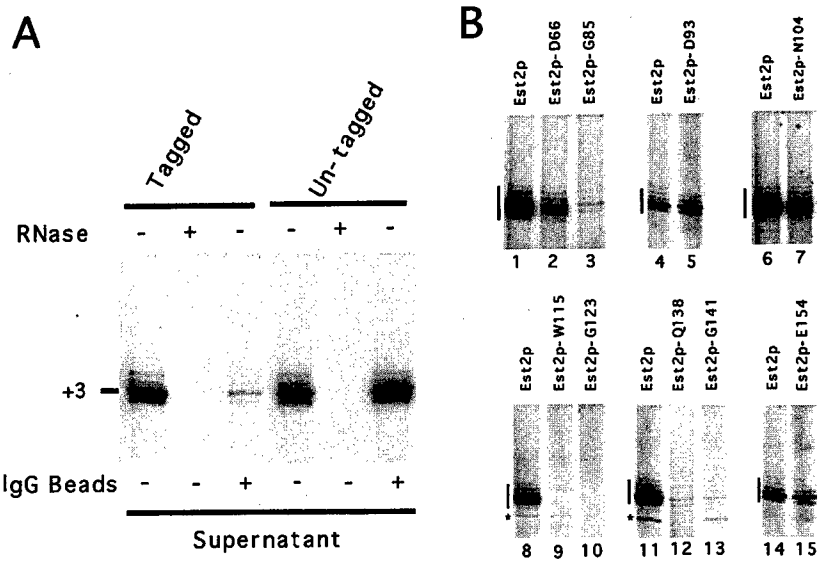

FIG. 3. Telomerase primer extension assays for wild-type and mutated RNPs. (A) The DEAE fraction was prepared from strains whose Est2p was either tagged or untagged with two copies of the IgG binding domain from protein A. The fractions were incubated either in the absence (−) or in the presence (+) of IgG-Sepharose beads. The supernatants were then recovered, incubated in the absence (−) or the presence (+) of RNase A, and assayed for telomerase activity using TEL66 (TAGGGTAGTAGTAGGG) as the primer oligonucleotide. The position of the primer +3 products is marked to the left of the panel. (B) DEAE fractions were prepared from strains bearing wild-type and mutated Est2p. Each mutant fraction was tested alongside the wild-type fraction using equal amounts of total protein. Each panel presents results from a single set of assays. The identities of the mutations are indicated at the top of each panel, and RNase-sensitive signals derived from telomerase are indicated by vertical bars to the left of each panel. An RNase-insensitive band (indicated by an asterisk) is occasionally observed in some assays.

Est2p. An immunoreactive species of ~115 kDa can be detected in extracts from the tagged strain, but not from the untagged strain, supporting the specificity of our assay (Fig. 5A). As expected, the two functionally defective mutants that exhibited wild-type levels of in vitro telomerase activity (D66A and N104AV105A) had wild-type levels of Est2p (data not shown). Four mutants with reduced telomerase activity in vitro (G85A, Q138AF139A, G141A, and N-10) also exhibited levels of Est2p comparable to that of the wild-type strain (Fig. 5B). The 12-fold or greater loss of in vitro activity of these four mutants was therefore not due to reduced Est2p expression-stability. Interestingly, the four senescent mutants (W115A,

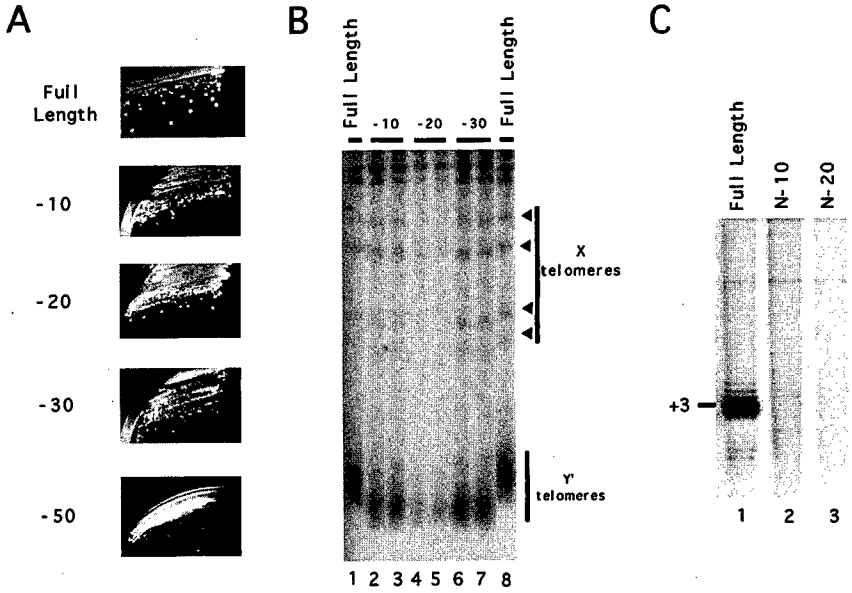

FIG. 4. N-terminal deletions result in defective Est2p function. (A) Transformants bearing either wild-type or N-terminally deleted Est2p were restreaked twice and monitored for growth defects on minimal plates. The photographs show colonies from the second restreak after 2 days of growth. The identities of the clones are indicated at the left of each panel. (B) Transformants bearing either wild-type or N-terminally deleted Est2p were restreaked twice, and single colonies were picked for growth in liquid cultures. Chromosomal DNAs were isolated from the cultures, digested with *Xho*I electrophoresed into a 1% agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled poly(dG-dT). The locations of the Y′ telomeres and X telomeres are indicated by a vertical bar and several arrowheads to the right of the panel, respectively. (C) DEAE fractions were prepared from strains bearing wild-type and N-terminally deleted Est2p and tested for telomerase primer extension activity using TEL66 as the primer oligonucleotide.

TABLE 2. Summary of in vivo and in vitro phenotypes of N-terminal deletions of Est2p

Residues deleted	Senescence[a]	Telomeres[b]	Primer extension activity[c]
None	−	+++	100
2–10	−	+/−	~2
2–20	−	+/−	~2
2–30	+	+/−	ND
2–50	+	+/−	ND

[a] Senescent strains (+) grew slowly on the initial transformation plate and gave rise to small colonies that are the same size as those from an Δ*est2* strain transformed with the pSE358 vector.

[b] Telomere lengths were determined in Southern hybridization assays (Fig. 2) and were scored as follows: +++, wild-type telomere length; +/−, on average about 300 bp shorter with weak hybridization signals.

[c] Primer extension assays were performed using TEL66 as primer under standard conditions. Each mutant fraction was assayed alongside the wild-type fraction (Fig. 3). The signals obtained from the mutant fractions were normalized against that from the wild-type fraction, which was taken as 100. ND, not determined.

F118AH119A, G123A, and N-30) did manifest a significant reduction in Est2p level such that it was not possible to detect these polypeptides unequivocally in unfractionated extracts (data not shown). However, following IgG-Sepharose purification, even these mutant proteins can be clearly visualized in Western analysis (Fig. 5C). The increased background in these latter assays (marked by a vertical bar to the right of the panel) came from IgG that was released by heating of the IgG-Sepharose beads in SDS and that reacted with the secondary antibody. Using signals derived from different amounts of Sepharose beads carrying wild-type Est2p as standards, these four mutant polypeptides appear to be present at approximately one-third to one-fifth of the wild-type protein level (compare lanes 3 to 6 with lane 1 and lane 2). The N-30 mutant polypeptide exhibited a slightly increased mobility by SDS-PAGE, further confirming the authenticity of our signal (Fig. 5C, lane 6). Given that telomerase activity was reduced by 50-fold or more in these senescent mutants, it appears that the mild reduction in Est2p level cannot solely account for the enzymatic defect.

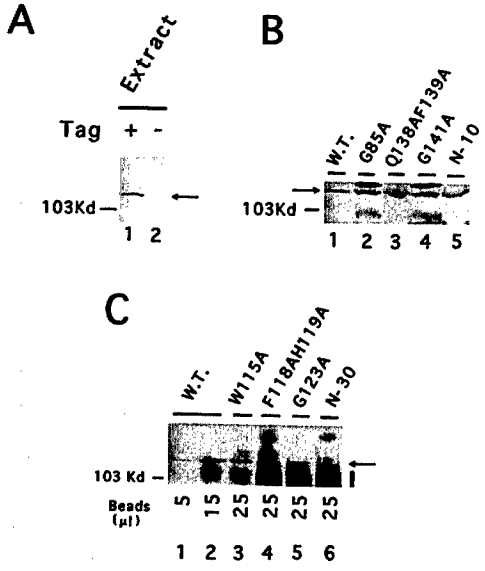

FIG. 5. Analysis of mutant protein expression. (A) Whole-cell extracts were prepared from strains whose Est2p was either tagged (+) or untagged (−) with the IgG binding domain of protein A and subjected to Western analysis. For each extract, 300 μg of total protein was examined. The position of the protein A-tagged Est2p is indicated by an arrow. (B) Est2p levels in whole-cell extracts from wild-type (W.T.) or mutant strains were analyzed by Western blotting. For each extract, 500 μg of total protein was examined. The position of the protein A-tagged Est2p is indicated by an arrow. (C) Est2p levels in whole-cell extracts from wild-type (W.T.) or mutant strains were assessed by affinity precipitation followed by Western analysis. For each extract, 4 mg of total proteins was incubated with 40 μl of IgG-Sepharose resin with gentle agitation at 4°C for 16 h. The beads were washed extensively, and the indicated amount of Sepharose was boiled in SDS-PAGE loading buffer. The eluted proteins were then subjected to immunoblotting. The position of the protein A-tagged Est2p is indicated by an arrow. The increased background (marked by a vertical bar) was due to IgG that eluted from the beads and that cross-reacted with the secondary antibody.

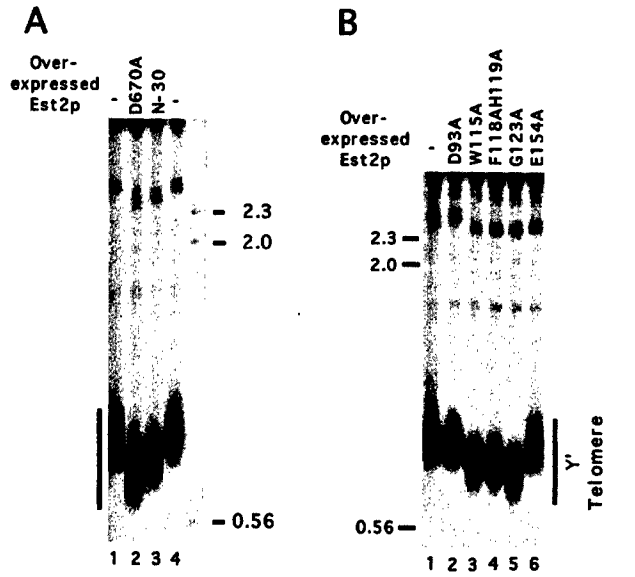

FIG. 6. Several Est2p mutants can cause telomere shortening when overexpressed in the presence of wild-type protein. (A) W303 was transformed with a pYX212 plasmid expressing mutated Est2p. After restreaking of the transformants three times, chromosomal DNAs were isolated from the strains, digested with *Pst*I, and analyzed for telomere lengths. DNAs were also isolated from untransformed W303 and tested for comparison. The source of the DNA is indicated at the top of the panel. (B) DNAs from strains overexpressing different Est2p mutant proteins were isolated and digested as for panel A and tested for telomere lengths. The source of the DNA is indicated at the top of the panel. The mobilities of several molecular size standards (in kilobases) are indicated at the sides.

If the senescent mutant proteins were indeed defective in function (as opposed to defective only in expression-stability), then they might act in a dominant-negative fashion when overexpressed in the presence of wild-type protein. This was found to be indeed the case. Four mutants that caused senescence (W115A, F118AH119A, G123A, and N-30) were placed downstream of a strong constitutive promoter, and the resulting plasmids were introduced into a wild-type strain (W303). Following three restreaks (~75 generations), chromosomal DNAs were isolated from the transformants and analyzed for telomere length alteration (Fig. 6). Consistent with a defect in function, all four mutants caused significant telomere shortening in the host strain (Fig. 6A, compare lane 3 with lanes 1 and 4; Fig. 6B, compare lanes 3 to 5 with lane 1). As expected, two mutant proteins that supported normal telomere maintenance (D93A and E154A) had no effect when overproduced (Fig. 6B, lanes 2 and 6), and an Est2p with an RT active site mutation (D670A) caused the most severe telomere shortening (Fig. 6A, lane 2). The other nonsenescent GQ motif mutants caused at most a slight shortening of telomeres (~50 bp) when overex-

pressed in W303, possibly because they retain a significant level of function (data not shown).

Identification of a protease-resistant stable domain in the N-terminal region of Est2p. To begin to biochemically dissect the TERT polypeptide, we attempted to express recombinantly the N-terminal region of Est2p in *Escherichia coli* as MBP (maltose-binding protein) fusion proteins. To facilitate purification, the proteins were also fused to a six-His tag at its C terminus. Three fragments, 1–304, 1–270, and 1–160, were chosen for initial characterization as parts of the MBP fusion protein [designated MBP-Est(1–304)p, MBP-Est2(1–270)p, and MBP-Est2(1–160)p, respectively]. Interestingly, the two larger fragments appear to be sensitive to proteolysis in *E. coli*. For example, following affinity purification over a maltose column, fractions derived from the MBP-Est2(1–304)p-overproducing strain contained not only the full-length fusion polypeptide but also several smaller fragments (Fig. 7A, lane 1). These smaller fragments most likely resulted from proteolysis of the Est2p segment in vivo, because they still retained the MBP domain, and because the MBP domain on its own was stably expressed in *E. coli* (Fig. 7A, lane 6). Based on their size, the proteolyzed fragments appear to retain ~160 amino acids of Est2p (Fig. 7A, compare lanes 1 and 2). Interestingly, the MBP-Est2(1–160)p can be easily overproduced and purified as a single polypeptide from *E. coli*, again suggesting that this segment of Est2p, encompassing the N region and GQ motif, can form a stable domain in vivo that is resistant to proteolysis.

The N-terminal domain of Est2p possesses a nucleic acid binding activity. One potential function for the N-terminal domain is involvement in protein-RNA interactions. To test this idea, we assayed the N-terminal fusion protein for RNA binding activity using a filter retention assay. Initial studies employed MBP-Est2(1–160)p and ^{32}P-labeled full-length TLC1 RNA. As shown in Fig. 7B, the amount of RNA retained on the filter increased with increasing protein concentrations. At the highest protein concentration used (0.05 μg/μl), ~25% of the input RNA was retained on the filter. The apparent dissociation constant was about 5 μM. The amount of RNA retained by MBP was substantially less than that by the fusion protein, indicating that RNA binding was mediated by the N-terminal Est2p fragment.

We investigated several reaction parameters and found that both salt and Mg^{2+} concentrations significantly affected the efficiency of binding. RNA retention as a function of Mg^{2+} concentration is a bell-shaped curve, with a peak at ~5 to 10 mM (Fig. 7C). The protein-RNA interaction was favored at low salt concentrations; the binding efficiency was reduced by ~75% at 250 mM sodium acetate relative to no salt (Fig. 7D).

To determine the sequence specificity of binding, we generated both sense and antisense TLC1 RNA probes by in vitro transcription and compared their abilities to interact with the fusion protein. As shown in Fig. 7E, the extent of binding is only twofold higher for the sense probe, suggesting that the N-terminal domain, at least by itself, does not recognize RNA with significant sequence specificity. We also tested the effect of RNA length on the efficiency of binding using TLC1 RNA missing increasing numbers of 3′-end residues. The results indicate that the fusion protein has similar affinities for RNAs ranging from 450 to 1,300 nucleotides long (data not shown).

We next tested binding of the fusion protein to DNAs of different structure using a competition filter-binding assay. A fixed amount of fusion protein [MBP-Est2(1–160)p, 1.3 μg] was mixed with both a fixed amount of labeled TLC1 RNA (10 ng) and variable amounts of unlabeled DNA competitors. The resulting mixture was then subjected to filtration through a nitrocellulose membrane as previously described. Single-stranded circular ϕX174 virion DNA, sheared and denatured salmon sperm DNA, and double-stranded linear DNA were used as the competitors. As shown in Fig. 7F, at an RNA/DNA ratio of 2:1, both the ϕX174 DNA and denatured salmon sperm DNA reduced the binding efficiency by 60%. Comparable inhibition of RNA binding by double-stranded linear DNA was achieved at a ratio of ~8:1. We also tested short single-stranded telomere oligonucleotides (the G-rich strand) in the competition assay, and the same amount of these oligonucleotides (by weight) was no more effective than the other single-stranded DNAs in reducing the binding signal (data not shown). Thus, the Est2p(1–160) fragment can also bind DNA in a non-sequence-specific fashion, with a slight preference for single-stranded DNA over double-stranded DNA.

The first 50 amino acid residues of Est2p are required for the nucleic acid binding activity. To further define the nucleic acid binding domain, we constructed a series of plasmids for expressing subfragments of the Est2p N-terminal domain. As before, these subfragments were fused to both an MBP and a six-His tag. The fusion proteins were expressed in and purified from *E. coli* as previously described (Fig. 7A) and used in filter binding assays.

As shown in Fig. 8A, a large deletion from the C-terminal end of the Est2p fragment had little effect on RNA binding. Even a fusion protein with only the first 50 amino acid residues of Est2p [MBP-Est2(1–50)p] showed binding to RNA comparable to that of MBP-Est2(1–160)p. In contrast, a 10-amino-acid deletion from the N terminus significantly reduced the RNA binding activity, by approximately fivefold. When 30 or 50 amino acids were deleted from the N terminus, the RNA binding activity was reduced to the background level (Fig. 8B; also see the MBP plot in Fig. 7B). A similar series of assays using a double-stranded linear DNA as the probe gave essentially identical results (data not shown). These observations suggest that the N region (first 50 amino acids) of Est2p is largely responsible for the nonspecific nucleic acid binding activity exhibited by the 1–160 fragment.

DISCUSSION

We have investigated the function of the N-terminal region of Est2p (yeast TERT) by a combination of HMM-based sequence comparison, mutagenesis, and biochemical studies. In this report, we describe the identification of four phylogenetically conserved non-RT motifs (named GQ, CP, QFP, and T) located in the N-terminal region of TERTs. The locations of these motifs within Est2p are as follows: GQ, residues 45 to 163; CP, residues 245 to 265; QFP, residues 267 to 343; and T, residues 367 to 413. Alanine substitutions of conserved GQ motif residues confirmed the functional importance of this motif. Indeed, the identification of two phenotypic classes of mutations within this motif suggests that it may mediate at least two distinct biochemical functions. Finally, we showed that the extreme N-terminal, nonconserved region (N region) of TERT possesses a nonspecific nucleic acid binding activity when analyzed in isolation. Furthermore, the N region proved to be important for telomere maintenance and telomerase activity in the context of the native telomerase RNP.

Comparison of the unigenic evolution approach and the HMM-based alignment approach in identifying functionally important TERT regions. While our work was in progress, Friedman and Cech (11) reported the identification of essential yeast TERT N-terminal domains using a unigenic evolution approach. In this approach, the gene of interest is heavily mutagenized, and functional variants are selected. Essential

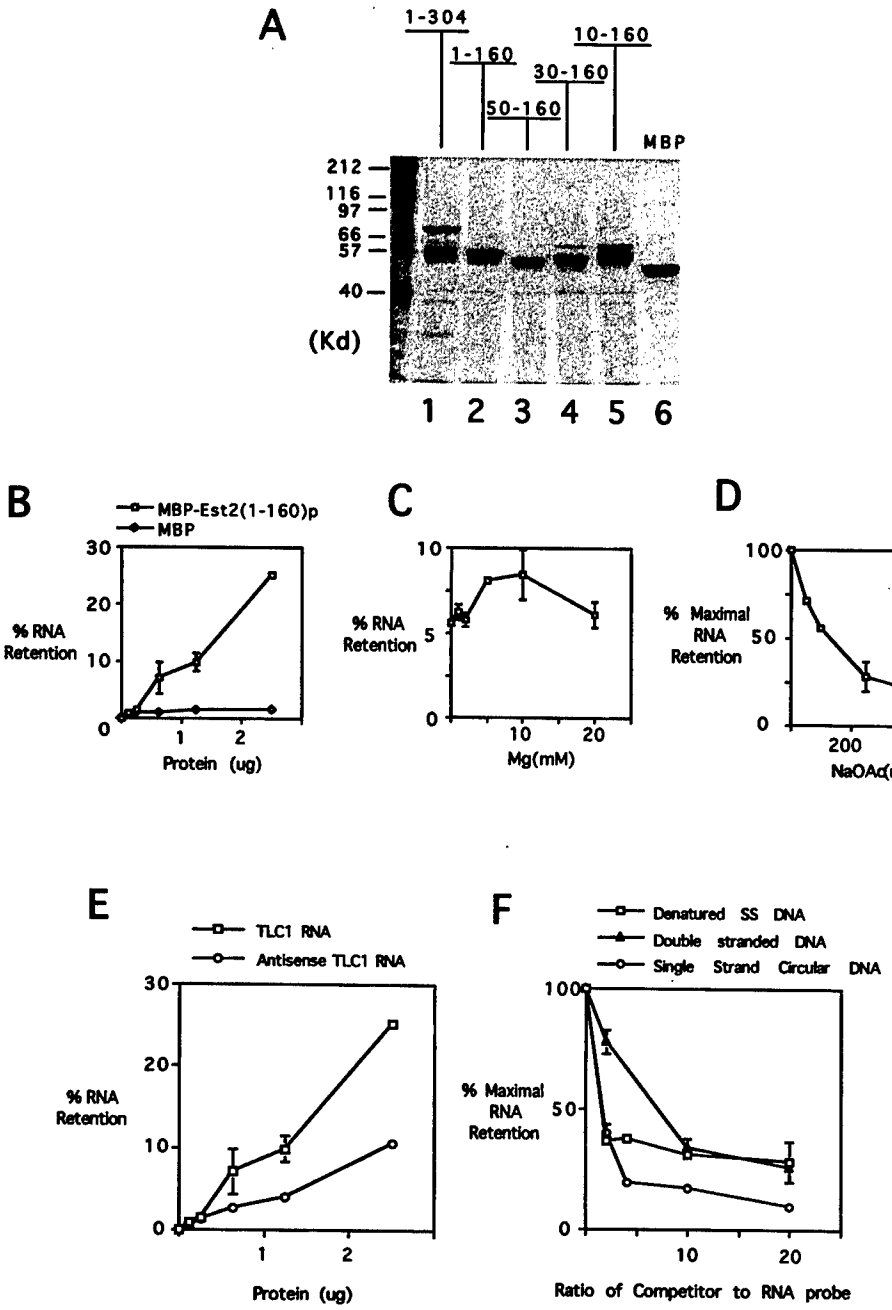

FIG. 7. The Est2(1–160)p fragment exhibits a nonspecific nucleic acid binding activity. (A) Several MBP-Est2p fusion proteins were expressed and purified from *E. coli* using both nickel-affinity and maltose-affinity columns. The resulting preparations were analyzed in an SDS–10% polyacrylamide gel. The identities of the fusion proteins are indicated at the top of the panel. All fusion proteins (lanes 1 to 5) contain ~40 kDa of an MBP domain besides the Est2p fragments. The MBP (lane 6) derived from the original cloning vector contains an extra protein fragment beyond the polylinker and is therefore ~48 kDa in size. (B) Increasing amounts of MBP and MBP-Est2(1–160)p were incubated with 20 ng of in vitro-transcribed, labeled TLC1 RNA. The resulting mixtures were passed through a nitrocellulose filter, and the percentage of probe retained on the filter was plotted against the amount of protein used. Assays were performed in duplicate, and the averages and spreads are indicated. (C) Filter binding assays were performed using 1.3 μg of MBP-Est2(1–160)p and 20 ng of TLC1 RNA in the presence of increasing Mg concentrations. The percentage of probe retained on the filter was plotted against the Mg concentration. (D) Filter binding assays were performed using 1.3 μg of MBP-Est2(1–160)p and 20 ng of TLC1 RNA in the presence of increasing sodium acetate concentrations. The percentage of probe retained on the filter was plotted against the sodium acetate concentration (taking the amount retained in the presence of no salt as 100%). (E) Filter binding assays were performed using increasing amounts of MBP-Est2(1–160)p and 20 ng of either TLC1 RNA or antisense TLC1 RNA probe. The percentage of probe retained on the filter was plotted against the amount of protein used. (F) Filter binding assays were performed using 1.3 μg of MBP-Est2(1–160)p and 10 ng of labeled TLC1 RNA in the presence of increasing amounts of three different unlabeled DNA competitors: denatured salmon sperm DNA, double-stranded linear DNA, and single-stranded (SS) circular DNA. The percentage of probe retained on the filter was plotted against the ratio of competitor to probe (taking the amount retained in the presence of no competitor as 100%).

and dispensable regions of the protein are then identified by statistically analyzing the distribution of missense and silent mutations. This analysis led to the identification of four essential regions in the Est2p N-terminal portion: region I, residues 31 to 163; region II, 214 to 265; region III, 285 to 374; and region IV, 378 to 432. A casual comparison between these regions and our motifs immediately indicates that they are largely concordant, with the GQ motif corresponding to region

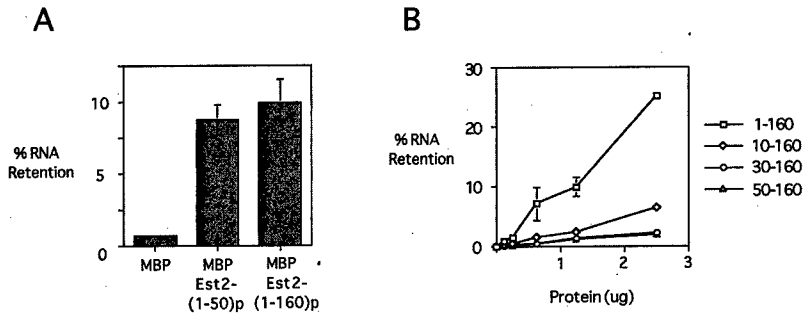

FIG. 8. The first 50 amino acid residues of Est2p are largely responsible for the nucleic acid binding activity exhibited by the N-GQ fragment. (A) Filter binding assays were performed using 1.3 μg of MBP, MBP-Est2(1–50)p, and MBP-Est2(1–160)p and 20 ng of labeled TLC1 RNA. The percentage of probe retained on the filter for each protein was plotted. (B) Filter binding assays were performed using increasing amounts of N-terminally truncated fusion proteins and 20 ng of labeled TLC1 RNA. The percentage of probe retained on the filter was plotted against the amount of protein used.

I, the CP motif corresponding to region II, and the QFP and T motifs corresponding to regions III and IV, respectively (Fig. 1B).

Because the unigenic evolution approach is aimed at identifying all essential regions, one would predict that the conserved motifs should constitute subparts of these regions. This is indeed the case for both the GQ and CP motifs. However, in the case of QFP and T motifs, there are two evident discrepancies that are worth noting. First, the QFP motif appears to encompass more residues at its N-terminal boundary than does region III. Nevertheless, mutations that are tolerated in the spacer between region II and region III do not affect conserved residues. Second, the boundary between region III and region IV and that between motif QFP and motif do not correspond. However, as noted by Friedman and Cech (11), because of the close proximity of these two regions, the choice of boundary is somewhat arbitrary.

While we did not present mutagenesis of conserved CP, QFP, and T motif residues in this report, preliminary studies indicate that conserved residues within these motifs are also required for normal telomere maintenance (Y. Peng and N. Lue, unpublished data). Furthermore, close inspection of tolerated mutations reported by Friedman and Cech (11) lends further credence to the validity of the alignment. Out of the 166 tolerated missense mutations, only 14 affect conserved or nearly conserved residues, and out of the 14 mutations, only 5 (N80→D, A360→E, T384→A, T384→I, and E385→V) change a conserved residue to a chemically dissimilar one. The identification of physiologically important conserved motifs that account for much of the N-terminal portion of TERTs suggests that this region shares a common structure and mediates conserved functions in telomere maintenance.

The function of the GQ motif. While mutagenesis indicates that the GQ motif mediates an important function(s), its precise mechanisms are not understood. As described earlier, two mutations in nonconserved residues failed to affect telomerase function in vitro and in vivo. In contrast, eight out of eight mutants with changes in conserved residues show defective telomerase function in vivo. These mutations can be further classified according to the level of telomerase primer extension activity in vitro: one class of mutants (called class A, six of eight mutants) has 1/12 or less the wild-type levels of activity, while the other class (called class B, D66A and N104AV105A) has nearly wild-type levels of activity. The identification of phenotypically distinct mutants suggests that the GQ motif may play at least two functions in telomere maintenance. Potential defects for the two classes of mutants are discussed separately below.

For class A mutants, there is a good correlation between telomere shortening and loss of telomerase activity. For example, the G85A, Q138AF139A, and G141A mutants, which exhibit ~1/12 to 1/20 of the wild-type activity, have telomeres that are on average ~150 bp shorter and do not exhibit signs of senescence. The senescent strains (W115A, F118AH119A, and G123A) have nearly undetectable levels of telomerase activity (>50-fold reduction) and even shorter telomeres. Taken together, these results are consistent with the prevailing model that the equilibrium telomere length is established by both lengthening and shortening mechanisms, with the level of telomerase activity being a key component of the lengthening mechanism (32).

Since class A mutant proteins are present at normal or slightly reduced levels, their defects in telomere maintenance and telomerase activity cannot be explained by loss of expression or stability. Two possibilities can be considered. First, the assembly of Est2p into the telomerase complex may be affected. Consistent with this hypothesis, Friedman and Cech (11) found that two of their alanine substitution mutants (Ala-4, which changes residues 110 to 119 all to alanine, and Ala-5, which changes residues 145 to 154 all to alanine) exhibited reduced binding to TLC1 RNA. In this regard, it is also interesting to note that the region of hTERT (1 to 200) hypothesized to bind Hsp90 and P23 contains the GQ motif (21). Binding to Hsp90 and P23 has been suggested to play a role in the assembly of human telomerase RNP. It is possible that some of the class A mutations may disrupt Est2p interaction with Hsp90 and/or P23, thereby causing a reduction in telomerase activity. However, it should be noted that the ability of the four senescent mutants to act in a dominant-negative fashion suggests that the mutant proteins are capable of interacting with some component of the telomere pathway and interfering with its action. Second, it is possible that some of the class A residues participate directly in telomerase primer extension. Interestingly, secondary structure predictions suggest that the residues mutated in the senescent strains (W115A, F118AH119A, and G123A) may lie on the same face of an alpha helix and may therefore mediate the same function (I. Saira Mian, unpublished data).

Because the class B mutants (D66A and N104AV105A) have wild-type levels of telomerase activity, the synthesis and assembly of the RNP are probably not affected. Instead, functional interaction between Est2p and other factors that regulate telomerase in vivo may be disrupted. Three groups of factors are potential candidates for this interaction based on epistasis analysis: Est1p and Est3p, the Rad50-Mre11-Xrs2 complex, and factors in the Tel1p-Tel2p pathway. Strains that

contain mutations in both telomerase core components (Est2p and TLC1) and factors in one of these three groups do not exhibit more severe growth defects or telomere shortening than do strains that carry single mutations (24, 40, 44). Thus, these factors may functionally interact with telomerase core components. Est1p and Est3p are presumed components of the telomerase holoenzyme but neither is required for in vitro activity. While the function of Est3p is not known, recent studies suggest that the Est1p may be involved in the recruitment of the core telomerase to chromosomal ends (9). The Rad50-Mre11-Xrs2 complex participates in nonhomologous double-stranded DNA break end-joining repair and possesses a 5′-to-3′ exonuclease activity (17). Tel1p is homologous to DNA-dependent protein kinases and other kinases involved in cell cycle checkpoint control (14, 36). Tel2p is an essential protein that exhibits a telomere sequence-specific DNA and RNA binding activity in vitro (23, 45). The mechanisms of these non-Est factors in telomere maintenance are poorly understood. Regardless of the precise interaction partner for the Est2p GQ motif, the fact that disruption of interaction can be caused by mutations in conserved residues suggests that the interaction may also be evolutionarily conserved.

The function of the N region. Our results suggest that the first 50 amino acid residues of Est2p (N region) are required for telomere maintenance in vivo and telomerase activity in vitro. These 50 amino acid residues, when separated from the rest of the RNP, are also capable of mediating nucleic acid binding in vitro. Deleting the first 10 amino acids of Est2p results in significant telomere shortening and an ~50-fold reduction in telomerase activity. The same deletion reduced the binding activity of the 1–160 fragment by fivefold. Deleting the first 30 or 50 amino acids had more severe effects both on the function of the full-length protein and on the binding activity of the 1–160 fragment. The correlation between function of the full-length protein and the binding activity of the N-terminal fragment suggests that the binding activity reflects an important physiologic function. We have been unable to express and purify a significant amount of full-length Est2p. Thus, it is not possible to compare the binding properties of N-terminal domain with those of the full-length protein. However, given the localization of the RT motifs in the C terminus of Est2p, the N-terminal domain is unlikely to account completely for the binding activity of the full-length protein.

The physiologic binding target for the Est2p N region is not clear. It seems unlikely that this fragment is binding both RNA and DNA nonspecifically in the context of the entire telomerase complex. Possibly, it has a specific binding target in vivo, but that it requires other domains of Est2p or other components of telomerase to achieve specific binding. One attractive target is the telomerase RNA. The N-terminal fragment may, for example, be involved in the formation of a stable RNP. This idea would be consistent with its important role in telomerase activity and telomere maintenance. Another attractive target is the telomeric DNA. TERT has been reported to possess a second primer-binding site away from the reverse transcription catalytic site (also known as the anchor site [7, 18]). The anchor site has been postulated to be important for processive elongation by ciliate telomerases. Earlier studies also suggest that anchor site interactions may be important for high-affinity primer-telomerase complex formation (29). Further studies will be necessary to define the precise target of the Est2p N region.

The N-GQ fragment may constitute a stable domain of TERT. Sequence analysis suggests that the conserved GQ motif is followed in the alignment by a presumed flexible spacer, which exhibits little conservation and is variable in length. This conjecture is supported by our expression studies with *E. coli*. As described in the Results section, while the 1–160 fragment can be easily overproduced and purified from *E. coli*, larger fragments are quite susceptible to proteolysis. In addition, unigenic evolution analysis indicates that this linker region may be largely dispensable for function. The sequence alignment, mutagenesis, and biochemical studies, taken together, suggest that all TERTs may have at least a bipartite domain organization, with conserved N-terminal (N-GQ fragment) and C-terminal (CP-QFP-T-RT fragment) domains connected through a flexible spacer. The identification of a stable domain of TERT should open the way toward detailed structural analysis of at least an important part of telomerase. Given that telomerase has been shown to be an attractive anticancer drug target, such a structural study will no doubt be interesting both from a biochemical and from a pharmacological perspective.

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J.X. and Y.P. contributed equally to the work.

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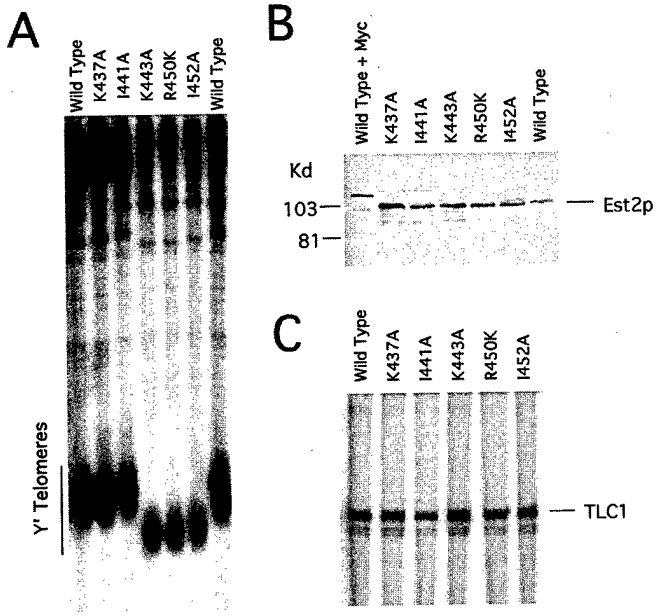

FIG. 2. **Telomere lengths, Est2p expression levels, and Est2p-associated telomerase RNA in the wild-type and mutant strains.** *A*, the lengths of telomeres in the wild-type and mutant strains were determined by Southern blotting. The locations of the Y′ class of telomeres are indicated on the left. *B*, Est2p protein levels in the wild-type and mutant strains were determined by Western blotting. The extract in the *first lane* was prepared from a strain in which Est2p was fused to both a 3-Myc tag and a protein A tag. The reduced mobility of the immunoreactive species detected in this lane indicates that the assays were correctly identifying Est2p. *C*, the levels of Est2p-associated *TLC1* RNA in the wild-type and mutant strains were determined by RNase protection assays. The position of the protected *TLC1* fragment is indicated on the right.

which contacts the templating and surrounding bases and the tip of which interacts with the deoxynucleotide triphosphate. Based on an alignment of HIV-1 RT and nine TERTs in this region (Fig. 1*A*), we chose 5 residues in Est2p for mutagenesis and detailed biochemical analysis: Lys^{437} (which is moderately conserved among TERTs, but not in RTs) and Ile^{441}, Lys^{443}, Arg^{450}, and Ile^{452} (which are conserved between TERTs and RTs). Of the four "universally" conserved residues, Lys^{443} (the equivalent of Lys^{65} in HIV-1 RT) and Arg^{450} (the equivalent of Arg^{72}) are presumed to contact the γ- and α-phosphates of the nucleotide substrate, respectively; Ile^{452} (the equivalent of Leu^{74}) is presumed to stack against the templating base; and Ile^{441} (the equivalent of Ile^{63}) is to make no direct contact with the substrates (Fig. 1*B*). Four of the five mutations (K437A, I441A, K443A, and I452A) are alanine substitutions, whereas one (R450K) is a conservative change, altering an invariant arginine to lysine. Each mutant was tagged at the C terminus with tandem copies of the IgG-binding domain of protein A, placed on a centromeric plasmid, and used to complement a yeast strain whose chromosomal *EST2* gene has been disrupted. A similarly tagged wild-type *EST2* gene was tested in parallel as the control.

Interestingly, only the K443A and R450K mutants failed to support normal yeast growth (data not shown). Strains carrying wild-type Est2p and the other three mutants grew normally on plates and in liquid medium. Analysis of telomere lengths yielded largely congruent results, with the K443A and R450K strains exhibiting severe telomere shortening; the I452A strain exhibiting moderate shortening; and the K437A, I441A, and wild-type strains exhibiting normal telomere lengths (Fig. 2*A*).

Finger Domain Mutations Do Not Alter the Est2p Expression Level or the Level of Associated Telomerase RNA in Vivo—To determine whether altered protein expression can explain the physiologic defects observed in the finger mutants, we prepared extracts from the respective strains and performed immunoblotting studies using antibodies directed against the protein A tag. As show in Fig. 2*B*, approximately equivalent amounts of the wild-type protein and each of the mutant proteins were detected in the assay, indicating no defect in protein expression. Because the finger domain has been implicated in RNA binding, we also measured the level of Est2p-associated telomerase RNA (*TLC1*) using an RNase protection assay. As shown in Fig. 2*C*, nearly identical amounts of *TLC1* RNA were obtained from each extract in the IgG-Sepharose precipitate, consistent with normal ribonucleoprotein formation in both the wild-type and mutant strains. These results are in agreement with previous studies indicating that stable TERT-RNA binding is mediated primarily by N-terminal TERT-specific motifs that lie outside of the RT domain (38–40).

Nucleotide-binding Residues Are Required for Normal Levels of in Vitro Telomerase Primer Extension Activity—The normal levels of Est2p and Est2p-associated *TLC1* RNA suggest that the functionally defective finger mutants may have enzymatic deficiency. To address this possibility, protein A-tagged telomerase from the wild-type and mutant strains was affinity-purified by specific adsorption to IgG-Sepharose and tested in primer extension assays (32, 41). Previous studies indicate that the tag has no effect on telomerase function and that labeled products derived from this procedure are almost entirely sensitive to RNase pretreatment, a hallmark of telomerase. Two primers were utilized for the assays: TEL15 (TGTGTGGTGTGTGGG), which consists of canonical yeast telomere repeats, and TEL66 (TAGGGTAGTAGTAGGG), which consists of heterologous repeats. The heterologous primer binds telomerase less stably, but supports greater overall DNA synthesis, most likely because of higher enzyme turnover (35). In addition, different combinations of labeled and unlabeled nucleotides (present at 0.2 and 33 μM, respectively) were used to differentiate between general and nucleotide-specific effects of mutations. (It was necessary to keep the labeled nucleotide at relatively low concentrations to maximize its specific activity).

As shown in Figs. 3 and 4, in standard reactions, the K443A and R450K mutants exhibited the greatest reduction in overall DNA synthesis. The defects were observed regardless of the primer or combination of labeled and unlabeled nucleotides. However, the two mutations appear to have a slightly different impact on nucleotide usage. With the TEL15 oligonucleotide as primer, the K443A mutant reproducibly supported greater DNA synthesis in the presence of low dTTP concentrations, whereas the R450K mutant was slightly more active in the presence of low dGTP concentrations (Fig. 4*A*). A more extensive set of assays was carried out using the TEL66 primer and varying concentrations of labeled dGTP or labeled dTTP (at 0.2, 0.7, and 3.2 μM) (Fig. 4, *B* and *C*). At higher concentrations of labeled nucleotides, the K443A and R450K mutants supported significantly higher levels of DNA synthesis (by as much as 7–10-fold). However, both mutants continued to exhibit the most severe defects in overall activity and to exhibit different preferences for low concentrations of dGTP and dTTP. These results imply a critical role for Lys^{443} and Arg^{450} in yeast telomerase activity and nucleotide utilization, consistent with their putative function in contacting (and perhaps positioning) the nucleotide substrate. The loss of overall activity upon mutating these residues is also consistent with the observed telomere maintenance defects.

In contrast to K443A and R450K, the K437A, I441A, and I452A mutants supported nearly normal levels of DNA synthesis, such that the increase or decrease was no more than 50%

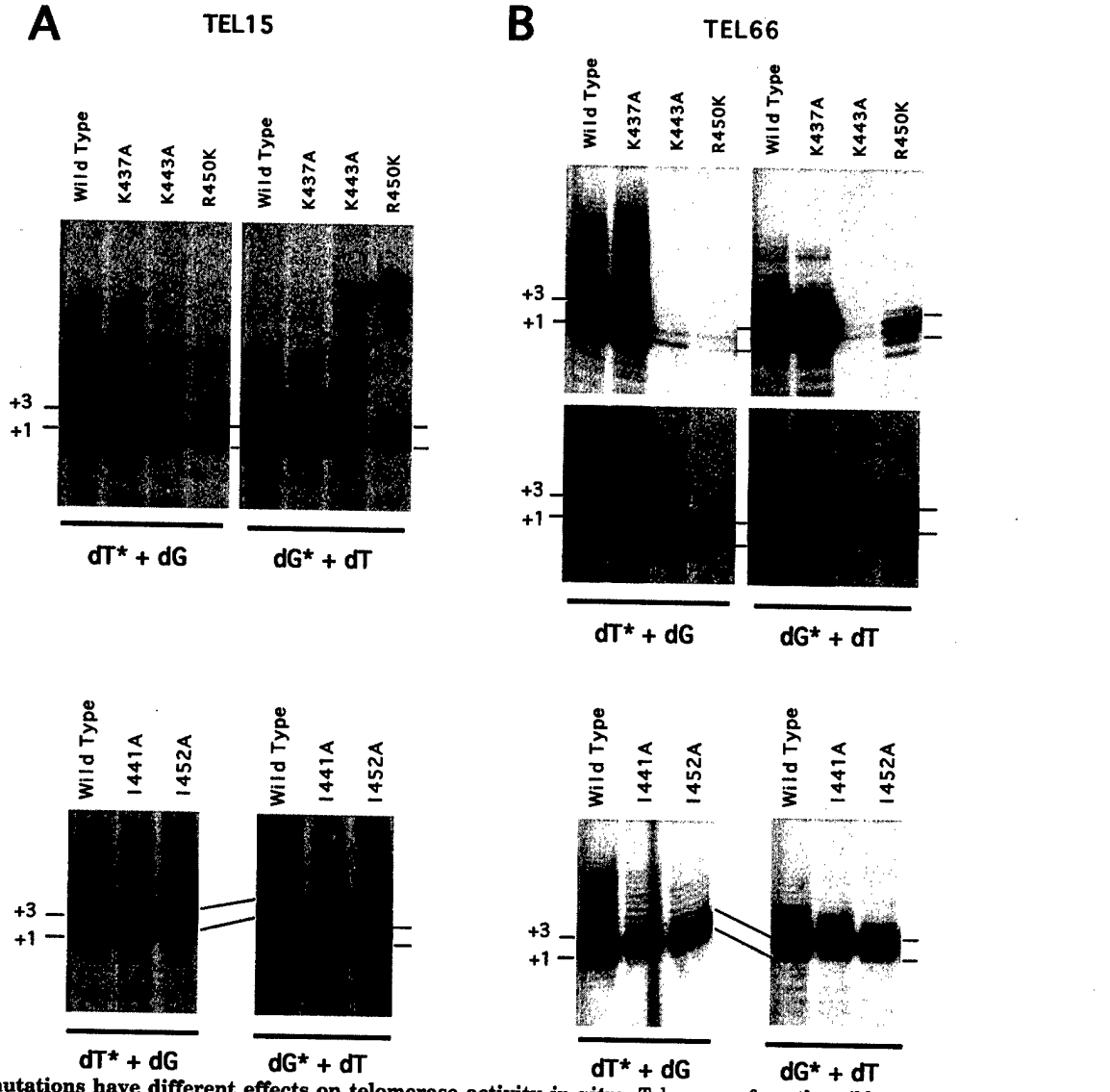

FIG. 3. **Finger mutations have different effects on telomerase activity *in vitro*.** Telomerase from the wild-type and various mutant strains was isolated by IgG affinity chromatography and tested in primer extension assays using either the TEL15 primer (*A*) or TEL66 primer (*B*). The identities of the extracts are indicated above, and the combinations of labeled nucleotides (indicated by *asterisks*; present at 0.2 μM) and unlabeled nucleotides (present at 33 μM) are indicated below. The locations of the P+1 (*+1*) and P+3 (*+3*) products are indicated. Two exposures are shown for the assays using the wild-type, K437A, K443A, and R450K extracts and the TEL66 primer to better illustrate the quantitative difference in activity among these extracts.

compared with the wild-type enzyme (Figs. 3 and 4). This observation held true regardless of the primer or combination of labeled and unlabeled nucleotides. The moderate decrease in overall activity of the I452A mutant (in assays utilizing the TEL66 primer) appears to be insufficient to account for its telomere maintenance defect, especially in light of the nearly normal telomere length of the I441A mutant, which exhibited a comparably moderate decrease in overall activity. The I452A mutant may therefore suffer from a defect that is not readily apparent in total activity measurements (see below).

Nucleotide- and RNA-binding Residues Are Required for Normal Telomerase Processivity in Vitro—Several nucleotide- and RNA-binding residues in the finger domain of HIV-1 RT have been implicated in processivity control (51, 52). To investigate if this is applicable to telomerase, we quantitatively determined processivity of the wild-type and mutant enzymes at multiple positions along the template. Because the primer used for these assays (TEL66) ends in 3 G residues, it can align only with the RNA template in one registry, allowing the addition of a defined sequence (TGTGGTG). In turn, this enables one to determine the amount of transcripts at each extension position (by normalizing the intensity signals to the number of labeled residues) and consequently processivity at each position. Different combinations of labeled nucleotide (present at 0.2, 0.7, and 3.2 μM) and unlabeled nucleotide (present at 33 μM) were tested to differentiate between general and nucleotide-specific effects of mutations on processivity. For ease of description and visualization, only processivity at selected positions near the start of extension is plotted in Fig. 5.

Several general observations can be made through this quantitative analysis. First, the absolute value of processivity is dependent upon the extension position along the RNA template. Second, the effects of mutations on processivity are position-specific. Third, the nucleotide concentration dependence of processivity is position-specific. All these observations are consistent with previous studies (29),[2] and with the notion that telomerase may exhibit conformational heterogeneity as its

[2] D. Bosoy and N. Lue, submitted for publication.

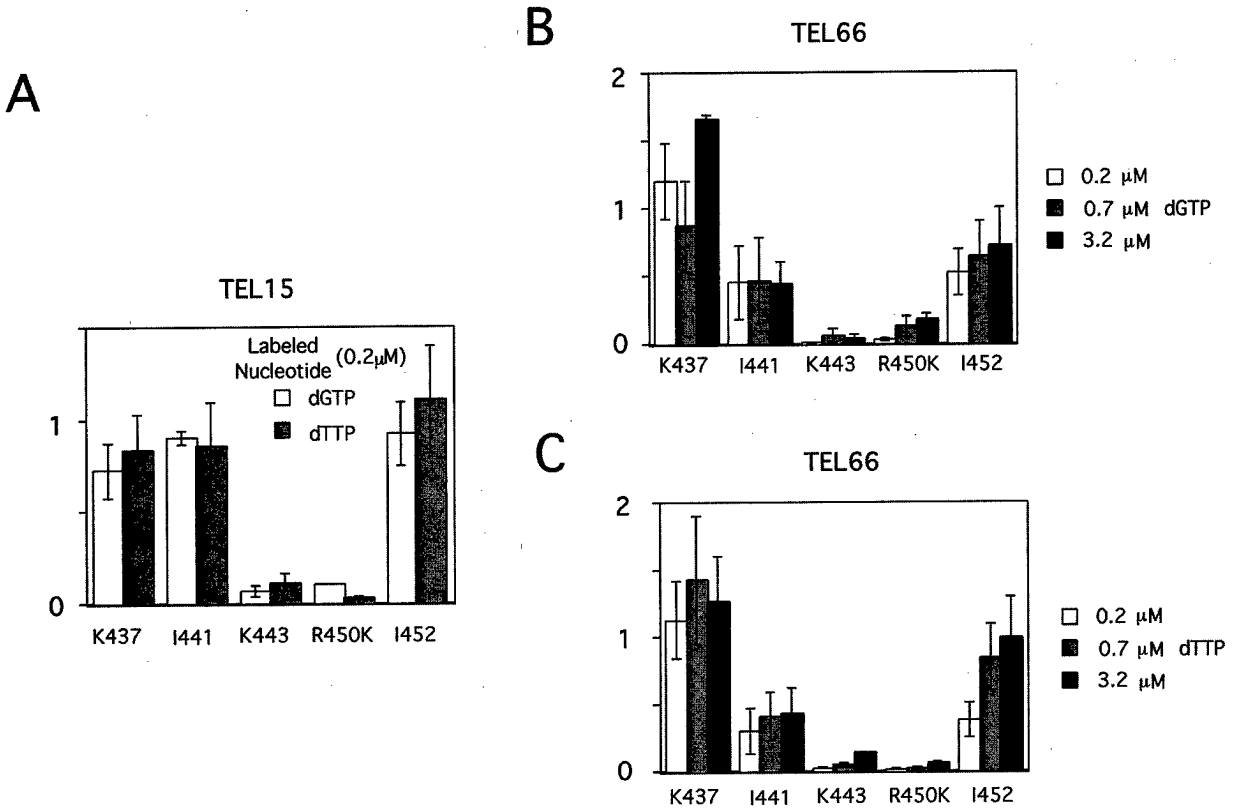

FIG. 4. **Effects of Est2p mutations on overall DNA synthesis *in vitro*.** *A*, the overall levels of DNA synthesis mediated by the mutant enzymes relative to wild-type telomerase using the TEL15 primer are plotted. Two different combinations of labeled and unlabeled nucleotides were tested, with the labeled nucleotide being present at 0.2 μM. *B*, the overall levels of DNA synthesis mediated by the mutant enzymes relative to wild-type telomerase using the TEL66 primer in the presence of increasing concentrations of labeled dGTP are plotted. *C*, the overall levels of DNA synthesis mediated by the mutant enzymes relative to wild-type telomerase using the TEL66 primer in the presence of increasing concentrations of labeled dTTP are plotted.

active site moves along the RNA template, resulting in different extension properties (*e.g.* processivity and rate constant) at different positions.

Different mutations in the putative finger domains of telomerase caused distinct processivity defects. The two mutants that exhibited the shortest telomeres and the lowest levels of total activity (K443A and R450K) also had the most severe processivity defects. The defects were especially evident at the P+2 position (where P is primer; often a 2-fold or greater loss of processivity) and were present regardless of the combination of nucleotides used (Fig. 5, *B* and *C*). Increasing the concentration of the labeled nucleotide from 0.2 to 0.7 or 3.2 μM failed to improve the processivity of these two mutants. The other mutant with greatly shortened telomeres (I452A) had a milder processivity defect that was especially evident at the P+3 position in the presence of labeled dGTP and unlabeled dTTP. In contrast to the K443A and R450K mutants, the processivity of the I452A mutant could be significantly improved by increasing the concentration of the labeled nucleotide (by more than 2-fold) (Fig. 5, *B* and *D*). The two mutants with no telomere maintenance defects (K437A and I441A) also exhibited slight reductions in processivity at certain positions with some combinations of nucleotides (*e.g.* the I441A mutant at the P+2 position in 0.2 μM dTTP). However, neither showed significant defects when the labeled nucleotide was present at 3.2 μM.

One Mutation Specifically Alters the Functional Interaction between the Nuclease and Polymerizing Activities of the Telomerase Complex—A relatively unique property of telomerase, among the family of RTs, is its ability to carry out primer cleavage. This nuclease activity appears to be conserved through evolution, although its physiologic function is not well understood. The activity is displayed by *Tetrahymena* telomerase reconstituted in rabbit reticulocyte lysate with just the TERT and RNA subunits and is thus likely to be mediated by one or both of these core components. In analyzing the primer extension activity of the telomerase mutants, we noticed that the R450K mutant appears to preferentially yield labeled products that can arise only from the combined action of nucleolytic cleavage and nucleotide addition. Specifically, with the combination of labeled dG and unlabeled dT, telomerase should incorporate radioactivity into the extension products starting at the P+2 position. Consistent with this expectation, the relative intensities of the P and P+1 products for wild-type telomerase and most of the telomerase mutants were quite low (Fig. 6*A*) (data not shown). In contrast, a significant fraction of the products for the R450K mutants migrated to the P and P+1 positions (marked by *triangles*), implying a relative increase in the action of the nuclease. Such an increase was not evident in the case of the K443A mutant (Fig. 6*A*). Quantitative analysis indicated that at low-to-moderate dGTP concentrations (0.2–3.2 μM), the fraction of the P and P+1 products was consistently higher (~5-fold) for the R450K mutant than for the wild-type enzyme (Fig. 6*B*). Indeed, with increasing dGTP concentrations, the relative intensities of the two shorter products became stronger, whereas the relative intensity of the P+3 product became weaker (marked by *diamonds*), suggesting a correlation between decreased processivity and increased cleavage. Preferential cleavage was not evident with the R450K mutant in the presence of labeled dT and unlabeled dG, possibly because the extremely low levels of DNA synthesis make it difficult to detect cleavage events (data not shown).

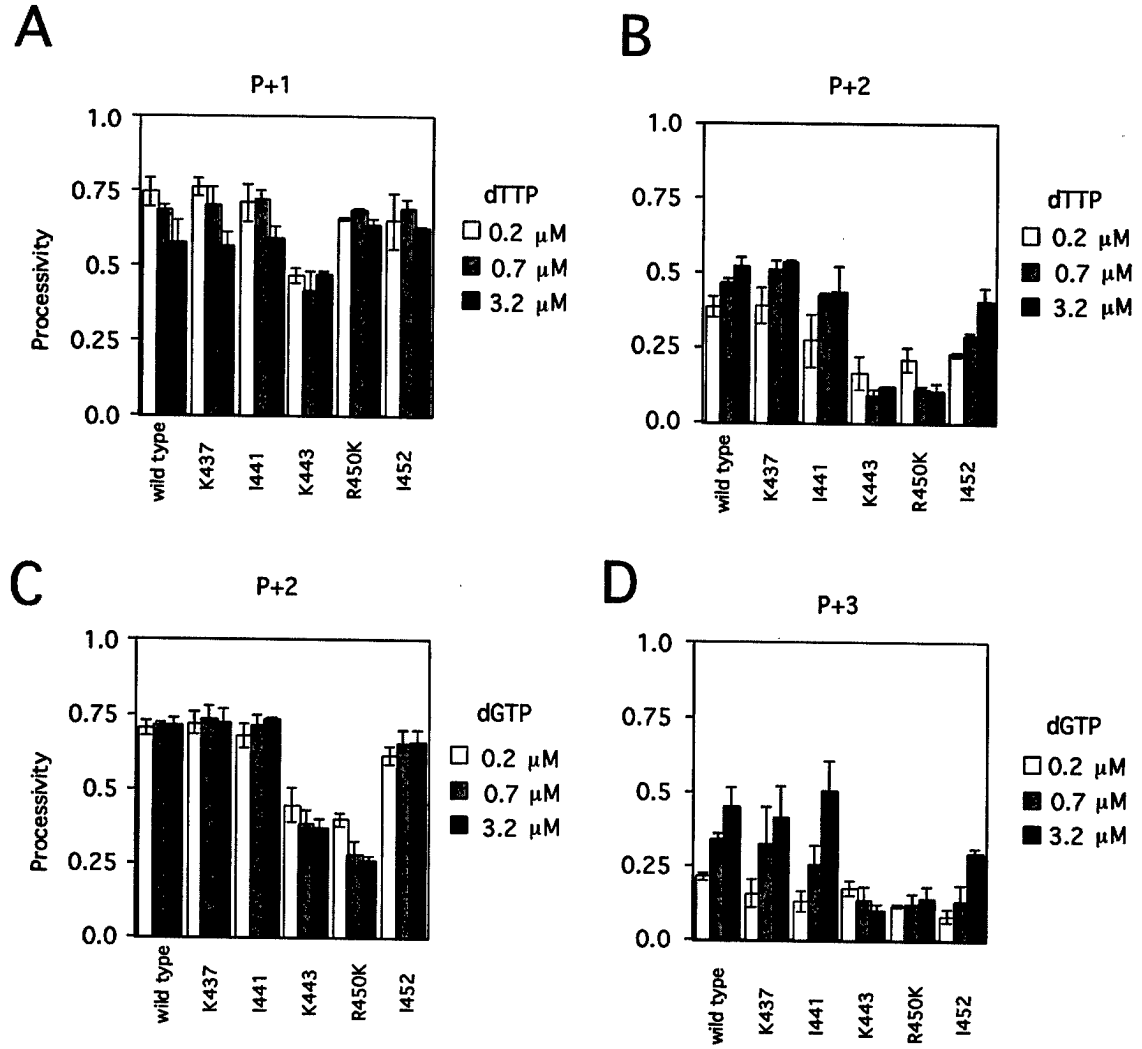

FIG. 5. **Effects of dGTP and dTTP concentrations on telomerase processivity at selected positions.** *A*, the processivity of wild-type and mutant telomerase at the P+1 position in the presence of increasing concentrations of labeled dTTP is plotted. *B*, the processivity of wild-type and mutant telomerase at the P+2 position in the presence of increasing concentrations of labeled dTTP are plotted. *C*, the processivity of wild-type and mutant telomerase at the P+2 position in the presence of increasing concentrations of labeled dGTP are plotted. *D*, the processivity of wild-type and mutant telomerase at the P+3 position in the presence of increasing concentrations of labeled dGTP are plotted. Processivity was determined from assays using the TEL66 primer.

DISCUSSION

Correlation between Sequence Conservation and Telomerase Function—Our results support the functional significance of sequence conservation between TERT and conventional RTs in the finger domain. In terms of physiologic requirement, residues absolutely conserved between TERT and other RTs are most essential (Lys^{443} and Arg^{450}); residues tolerant of conservative substitutions are less important (Ile^{441} and Ile^{452}); and a TERT-specific residue is more or less dispensable for telomerase function. The functional requirements are likely to be due to conserved structure and molecular mechanisms because residues believed to make direct contact with the substrates are shown to have greater importance both *in vitro* and *in vivo* (*e.g.* compare I441A and I452A mutants). Sequence alignment between TERT and conventional RTs thus appears to be an efficacious way of identifying important functional residues in telomerase.

Comparison of the Biochemical Defects of TERT and HIV-1 RT Mutants with Amino Acid Substitutions in the Finger Domain—Because both Lys^{443} and Arg^{450} are absolutely conserved and presumed to make direct contact with the nucleotide substrate, the impact of mutations of these residues on telomerase activity is readily comprehended. The K443A and R450K mutants exhibited a severe defect in both overall DNA synthesis and a defect in processivity at selected positions. Increasing the nucleotide concentration in the assays only slightly improved overall DNA synthesis without improving processivity. These results can be interpreted in terms of reduced binding of nucleotide and failure to properly position the nucleotide for polymerase chemistry. The differential effects of low dGTP and dTTP concentrations on the activity of the two mutants (Figs. 3 and 4) suggest that the mutations may differentially impact on the binding/positioning of these two nucleotides. Studies of the corresponding HIV-1 RT residues (Lys^{65} and Arg^{72}) yielded similar although not identical results (43, 44). Both residues are required for optimal total DNA synthesis and enzyme processivity. The K65A mutation caused differential alterations in the enzyme's K_m for different nucleotides, consistent with the mutation's having different impact on the binding of distinct nucleotides (43). However, the effect of alanine substitution at Lys^{65} on DNA synthesis and processivity appears to be much milder than that at Arg^{72} (44).

Alanine substitution of Ile^{452} in Est2p caused a slight defect in overall DNA synthesis and a moderate defect in telomerase processivity, both of which can be rationalized in terms of the location of the corresponding residue (Leu^{74}) in HIV-1 RT. In

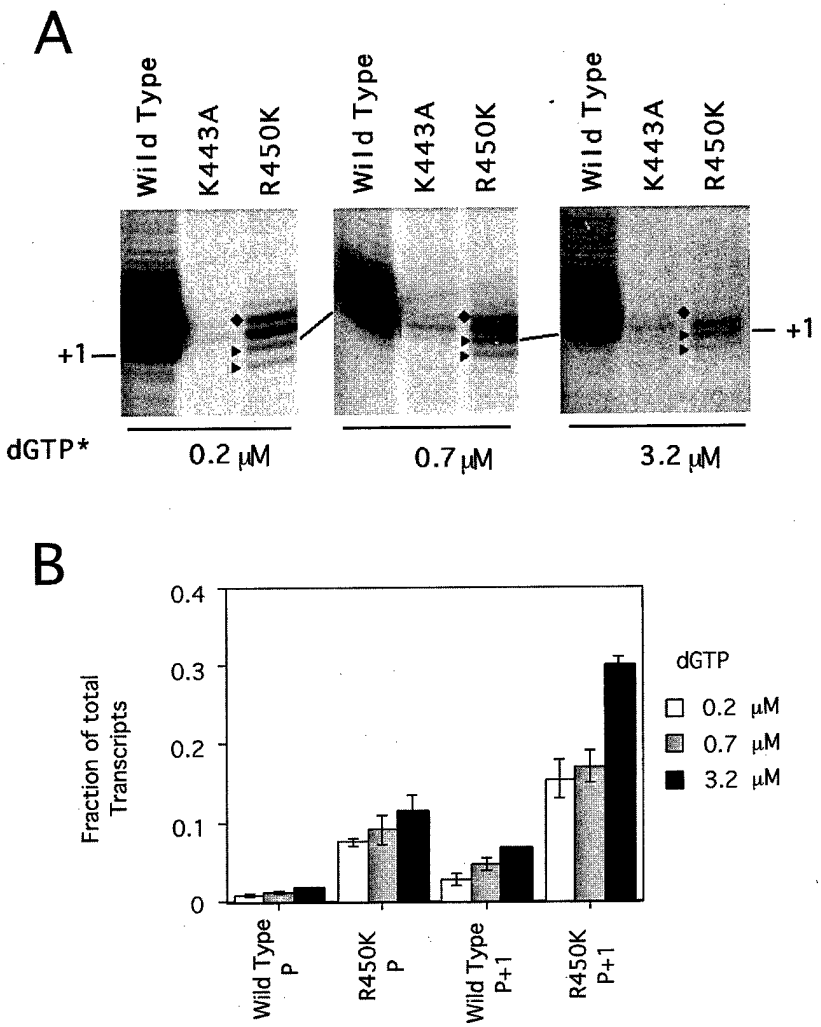

FIG. 6. **Effect of the R450K mutation on the primer cleavage activity of yeast telomerase.** *A*, the wild-type telomerase and the K443A and R450K mutants were tested in primer extension assays in the presence of three different concentrations of labeled dGTP. The positions of the P+1 (*+1*) product are indicated. *B*, the amounts of the P and P+1 products as fractions of total transcripts were determined from assays using three different dGTP concentrations and are plotted for both the wild-type enzyme and the R450K mutant.

the covalently trapped catalytic complex of HIV-1 RT, Leu^{74} appears to "lock the templating base tightly in place" (Fig. 1*B*) (31). The residue also contacts other side chains that bind dNTP directly. Thus, the defects of the I452A mutation may be due to altered template or nucleotide interactions. Indeed, a somewhat more conservative L74V substitution in HIV-1 RT has been shown to result in decreased processivity and resistance to didanosine (45), consistent with findings in TERT.

In contrast to Lys^{443}, Arg^{450}, and Ile^{452}, the residues in HIV-1 RT that correspond to Lys^{437} (possibly Pro^{59}) and Ile^{441} (Ile^{63}) do not appear to contact either the template or nucleotide. Under some *in vitro* assay conditions, both the K437A and I441A mutants can exhibit a slight defect in total DNA synthesis and/or processivity. These mutations may cause defects indirectly by altering the conformation or function of surrounding residues. These mild *in vitro* defects have no apparent physiologic manifestations under normal growth conditions.

Several conserved residues in the finger domain of *Tetrahymena* TERT have been analyzed by the reticulocyte lysate reconstitution system (30). In particular, residues corresponding to Lys^{437}, Lys^{443}, and Arg^{450} of Est2p have all been mutated and tested in this *in vitro* system (Lys^{532}, Lys^{538}, and Arg^{543} in *Tetrahymena* TERT, respectively). Curiously, in contrast to the yeast results, total nucleotide incorporation was not greatly reduced (<3-fold) by any of the *Tetrahymena* mutations. However, consistent with the yeast results, both the K538A and R543K mutants exhibited significant reductions in enzyme processivity, with the latter manifesting a specific inability to copy the 5′-end of the template.

Overall, this study of the finger domain mutations reveals a great deal of mechanistic conservation between telomerase and retroviral RTs. Other motifs/domains of TERT and retroviral RTs are also likely to be mechanistically similar according to earlier biochemical and genetic analysis of the palm and thumb domains of Est2p and biochemical analysis of *in vitro* reconstituted *Tetrahymena* and human telomerase (9, 18, 29, 30). Thus, despite the very limited sequence conservation, the molecular mechanisms of these two classes of RTs appear to be highly conserved. Because retroviral RTs and TERTs are evolutionarily distant, this conclusion further suggests that other classes of RTs (*e.g.* long terminal repeat retrotransposon RTs, non-long terminal repeat element RTs, and group II intron RTs) may be mechanistically quite similar as well (46, 47).

Interaction between the Nuclease and Reverse Transcriptase Activities of Telomerase—As described above, the R450K mutant appears to preferentially yield labeled products that can arise only from the combined action of nucleolytic cleavage and nucleotide addition. Although a number of telomerase RNA mutations have been reported to alter primer cleavage, this appears to be the first example of a protein mutation with such an effect. The greatly reduced processivity of the R450K mutant suggests a potential link between enzyme processivity and aberrant cleavage, as has been proposed earlier (48). However, the effect of the R450K mutation appears to be quite specific in that it is evident only with the combination of labeled dGTP and unlabeled dTTP nucleotides (Figs. 3 and 6). In addition, many other processivity mutants of Est2p do not manifest altered cleavage (*e.g.* a C-terminal truncation mutant) (29).

Despite a great deal of analysis, the molecular basis for the cleavage activity of telomerase remains elusive. The selective effect of a nucleotide-binding residue and the effects of many RNA template mutations on cleavage property support a close physical interaction between the two activities of telomerase (42, 49).

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