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Analysis of Telomerase Processivity: Mechanistic Similarity to HIV-1 Reverse Transcriptase and Role in Telomere Maintenance

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Summary

The key protein subunit of the telomerase complex, known as TERT, possesses a reverse transcriptase (RT)-like domain that is conserved in enzymes encoded by retroviruses and retroelements. Structural and functional analysis of HIV-1 RT suggests that RT processivity is governed, in part, by the conserved motif C, motif E, and a C-terminal domain. Mutations in analogous regions of the yeast TERT were found to have anticipated effects on telomerase processivity in vitro, suggesting a great deal of mechanistic and structural similarity between TERT and retroviral RTs, and a similarity that goes beyond the homologous domain. A close correlation was uncovered between telomerase processivity and telomere length in vivo, suggesting that enzyme processivity is a limiting factor for telomere maintenance.

Introduction

Telomerase is a ribonucleoprotein (RNP) that is responsible for maintaining the terminal repeats of telomeres in most organisms (Greider and Blackburn, 1985). It acts as a reverse transcriptase (RT), using a small segment of an integral RNA component as template for the synthesis of the dGT-rich strand of telomeres (Greider and Blackburn, 1989).

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 Blackburn, 1992; Bryan and Cech, 1999; Nugent and Lundblad, 1998). 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 exhibit no evident sequence similarity. A recent phylogenetic analysis of vertebrate telomerase RNAs suggests that they possess secondary structural elements present in the ciliate RNAs (Chen et al., 2000). The essential protein subunit of telomerase (TERT), initially purified from *Euplotes aediculatus* as p123, was later found to be homologous to Est2p, a yeast protein required for telomere maintenance (Lendvay et al., 1996; Lingner and Cech, 1996; Lingner et al., 1997b). Subsequently, homologs of TERT were identified in diverse organisms (Bryan et al., 1998; Collins and Gandhi, 1998; Fitzgerald et al., 1999; Greenberg et al., 1998; Kilian et al., 1997; Malik et al., 2000; Meyerson et al., 1997; Nakamura et al., 1997; Oguchi et al., 1999). Because coexpression of TERT and telomerase RNA in vitro in rabbit reticulocyte lysate (RRL) suffices to reconstitute enzyme activity (Beattie et al., 1998; Weinrich et al., 1997), these two subunits probably constitute the minimal functional core of the enzyme complex. In addition, both biochemical and genetic studies point to the existence of additional protein subunits of telomerase, whose functions remain to be elucidated. In particular, the yeast telomerase complex apparently contains two additional protein subunits (Est1p and Est3p), one of which may play a role in the recruitment of telomerase core to telomere ends in vivo (Evans and Lundblad, 1999; Hughes et al., 2000).

Sequence and functional analysis of cloned TERTs suggests a modular organization for the protein, with a central RT domain (possessing RT-like motifs) flanked by a long N-terminal extension, and a short C-terminal extension (Figure 1A). The N-terminal extension has been shown to encompass several conserved, TERT-specific motifs that are important for both the "enzymatic activity" and "regulation" of telomerase (Friedman and Cech, 1999; Xia et al., 2000). Mutations in the RT domain had detrimental effects on both telomere maintenance and telomerase activity, consistent with a direct catalytic role for this domain (Haering et al., 2000; Harrington et al., 1997; Lingner et al., 1997b; Weinrich et al., 1997). The C-terminal extension of TERT is also reported to be functionally important, though its precise role(s) is not understood (Friedman and Cech, 1999).

To date, mutational analysis suggests that the fundamental mechanisms of catalysis (e.g., the requirement for metal-coordinating acidic residues) are conserved between TERT and conventional RTs. However, the degree of conservation with regard to other aspects of polymerase mechanisms remains unclear. To address one component of this issue, we initiated a sequence alignment and structure-based analysis of telomerase processivity. Our approach is based on two previous lines of investigations: (1) molecular and biochemical analysis of processivity determinants in HIV-1 RT, and (2) the three-dimensional structural model of HIV-1 RT, which have been determined at high resolution in complexes with nucleic acids and nucleotide substrates (Ding et al., 1998; Huang et al., 1998). These earlier investigations revealed at least three important determinants of HIV-1 RT processivity, including the conserved RT motif C, motif E (also known as the "primer grip"), and a C-terminal domain (Figure 1A). In the three-dimensional structural model of HIV-RT, typically described as a right hand, the conserved motif C is located at the catalytic center within the "palm" domain. The conserved motif E corresponds to the β12-β13 hairpin (residues 227–235), located near the interface between the "palm" and "thumb" of the right hand. The C-terminal domain comprises a bundle of three α-helices (residues 237–317) constituting the "thumb". To determine if analogous

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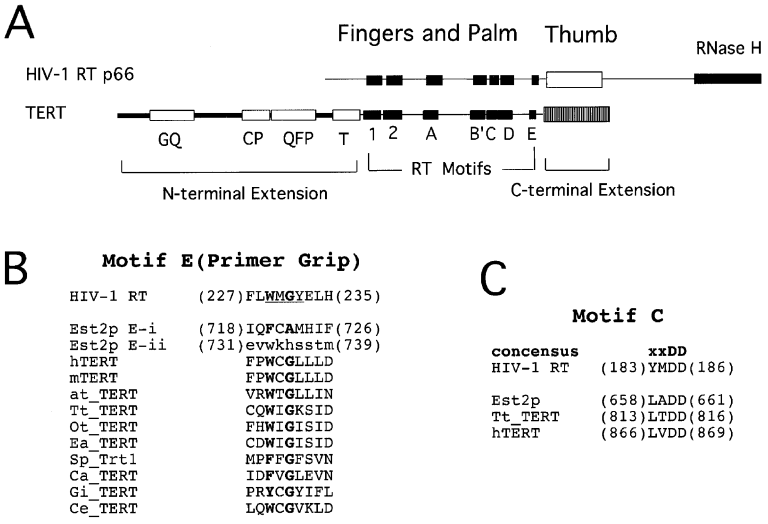

Figure 1. Organization of the p66 Subunit of HIV-1 RT and TERT, and Selected Alignments of Telomerase RT Motifs

(A) Regions of homology between the p66 subunit of HIV-1 RT and Telomerase Reverse Transcriptase. The conserved reverse transcriptase (RT) motifs (named 1, 2, A, B′, C, D, and E) are located in the N-terminal half of the HIV-1 RT and are often referred to as the fingers and palm of a right hand. This conserved region is followed by a thumb domain and an RNase H domain in HIV-1 polypeptide. In telomerase reverse transcriptase (TERT), the RT motifs are centrally located and flanked by a long N-terminal extension and a short C-terminal extension. The N-terminal extension appears to contain four evolutionarily conserved motifs (GQ, CP, QFP, and T).

(B) Primer Grips in HIV-1 RT and TERTs. An alignment of known and potential primer grips from these polypeptides is presented. The tip residues of the β-hairpin in HIV-1 RT are underlined. The third and fifth positions have been shown to be functionally important and are shown in bold.

(C) Alignment of part of motif C from HIV-1 RT and three TERTs.

structures exist in TERT, if they influence enzyme processivity, and if enzyme processivity affects telomere maintenance, we made point mutations and deletion mutations in motif C, E, and the C-terminal extension of yeast TERT (Est2p), and analyzed the phenotypic consequences. Our results suggest that with respect to processivity control, TERT is mechanistically very similar to HIV-1 RT, and probably to all other RTs. Furthermore, the data argue that the natural processivity of telomerase is a limiting determinant of the equilibrium length of telomeres in vivo.

Results

Identification of a Putative "Primer Grip" in the *Saccharomyces cerevisiae* TERT (Est2p)

A key determinant of HIV-1 RT processivity is the conserved RT motif E, or primer grip. To identify the structural counterpart in TERT, we undertook a comparative sequence analysis of all available TERTs, including the recently identified *Arabidopsis*, *Candida*, *Giardia*, and *Caenorhabditis* homologs. A variety of alignment algorithms, including one based on a Hidden Markov Model (HMM), identified residue 718–726 of Est2p (motif E-i) as homologous to residue 227–235 of the HIV-1 RT (Figure 1B). This alignment differs from several previously published ones, which postulates residue 731–739 (motif E-ii) to be the primer grip in Est2p (Bryan et al., 1998; Malik et al., 2000; Nakamura et al., 1997). The discrepancy may be due to the different number of TERT homologs included in the analysis, or the difference in alignment algorithms. Motif E-i has been independently identified as a potential primer grip in Est2p (A.M. Metz et al., submitted).

To determine if motif E-i or E-ii is important for telomerase function, as would be predicted for the true primer grip, we generated alanine substitution mutations in both regions and analyzed their effects on growth and telomere maintenance in vivo. As shown in Table 1, only the F720A mutation in motif E-i had an obviously detrimental effect on cell growth. The other two alanine substitutions in E-i (C721A and M723A) and the two mutations in E-ii (W733A and H735A) had little effect. However, analysis of telomere lengths by Southern blotting revealed significant differences between the E-i and E-ii mutants, with the E-i having telomeres that are shorter by 200 bps or more and the E-ii having nearly normal telomere lengths (Figure 2A, Table 1). The F720A mutant had the shortest telomeres, consistent with its growth defect.

The enzymatic defects associated with mutations in E-i and and E-ii were tested in primer extension assays using protein A-tagged telomerase that had been affinity purified by specific adsorption to IgG-sepharose (Friedman and Cech, 1999; Xia et al., 2000). Earlier studies showed that the tag has no effect on telomerase function, and that labeled products derived from this affinity procedure are almost entirely sensitive to RNase pretreatment, a hallmark of telomerase. Consistent with the telomere length assays, two E-i mutations (F720A and M723A) resulted in significantly reduced overall DNA synthesis by telomerase, while the remaining E-i mutation (C721A) and the E-ii mutations had minor (less than 2-fold) or no effect (Figure 2B, lanes 1–5). Western analysis of Est2p using antibodies directed against the protein A tag indicates that comparable amount of the various mutant proteins were present in the respective extracts, suggesting that enzyme function rather than stability was compromised by the mutations (Figure 2C).

The Function of the Est2p Primer Grip in Determining Telomerase Processivity In Vitro

Close inspection of the banding patterns of the in vitro reaction products suggests differences in the elongation

Table 1. Summary of In Vivo and In Vitro Phenotypes of Substitution and Deletion Mutants of TERT

Mutation	Complementation*	Telomeres#	Percentage Processivity Change (TEL15)**	Percentage Processivity Change (TEL66)**
None	+	++++	−	−
F720A	−	+	N.D.	N.D.
C721A	+	++	−36 (± 12)	−54 (± 9.4)
M723A	+	++	−28 (± 6.1)	−51 (± 15)
W733A	+	++++	−2.0 (± 3.2)	N.D.
H735A	+	++++	−3.9 (± 1.1)	N.D.
LA658YT	+	+	−39 (± 4.2)	−56 (± 13)
FCA720WCG	+	+++++	+24 (± 5.0)	+26 (± 10)
LA658/FCA720	+	++	−14 (± 4.8)	−18 (± 6.6)
C840	−	+	N.D.	N.D.
C800	−	+	N.D.	N.D.
C745	+	+	−44 (± 24)	−74 (± 3.8)
C715	−	+	N.D.	N.D.
IL290AA	+	++	+1 (± 10)	+5 (± 1.3)

* Est2p mutants that failed to complement the chromosomal EST2 disruption (−) gave rise to small colonies that are the same size as those from an Δest2 strain transformed with the pSE358 vector.

Telomere lengths were determined in Southern hybridization assays (Figure 2, 3, 5 and 6) and were scored as follows: +++++, on average about 50 bps longer; ++++, wild type telomere length; +++, on average about 50–100 bps shorter; ++, on average about 150–200 bps shorter; +, on average about 250–300 bps shorter.

** Processivity of telomerase from the primer+3 product to longer products was determined in triplicate experiments using TEL15 and TEL66 as primer oligonucleotides. Processivity of the mutant enzyme relative to the wild type enzyme was then calculated and both the average percentage change in processivity and the standard deviation (in parenthesis) reported.

property of the wild-type and mutated enzymes. For example, the intensity of the primer+4 product relative to that of the primer+3 product appears to be reduced for both the C721A and the M723A mutant (Figure 2B, lanes 1, 6, and 7). Earlier studies suggest that in standard yeast telomerase assays, all labeled products are likely due to a single cycle of binding and elongation (Prescott and Blackburn, 1997). To confirm this conjecture, a competition assay was performed, in which a test primer (TEL15) was pre-bound to telomerase, followed by the addition of excess competitor primer (TEL24) and nucleotides. As shown in Figure 2D, with this order of addition, the competitor primer had little effect on the banding pattern, consistent with the elongation products being due to processive DNA synthesis (compare TEL15 products in lanes 2 and 3 with lanes 4 and 5). When the competitor primer was added concurrent with the test primer, the products arising from the test primer were effectively abolished, as expected (lane 1). These results indicate that in standard reactions, telomerase is not capable of acting on an already extended and dissociated product. Several mutated telomerases were subjected to the same competition assay, with identical results (data not shown). Hence, alterations in the elongation pattern of the mutated telomerases can be attributed to changes in their processivity.

To determine processivity quantitatively, we calculated the relative amounts of the reverse transcripts at each extension position, and expressed processivity at each position as the fraction of transcripts that continued to elongate out of all transcripts that have reached a given position. Two primers were utilized in these assays: TEL15 (TGTGTGGTGTGTGGG), which consists of canonical yeast telomere repeats, and TEL66 (TAGG GTAGTAGTAGGG), which consists of heterologous repeats. As shown in Figure 2E, for both primers, the processivity exhibited by wild-type telomerase during elongation was quite reproducible, with standard deviations that range from 2%–13%. The processivity for the TEL15 primer was higher than the TEL66 primer at almost all positions, possibly reflecting the greater stability of the template-primer duplex mediated by the TEL15 primer. The processivity at the primer+3 position was low for the TEL15 primer (0.47) and was reduced further in the case of the TEL66 primer (0.18), suggesting that under the standard in vitro reaction condition, yeast telomerase has relatively more difficulty elongating beyond this position.

Quantitative comparison revealed clear processivity defects for telomerase with mutations in motif E-i. Consistent with the notion that the primer+3 position represents an elongation block for yeast telomerase, the processivity defects are most pronounced for the mutants at the primer+3 position (Figure 2F). The processivity values for the wild-type enzyme for TEL15 differ slightly between Figures 2E and 2F because they are from different sets of experiments. When TEL15 was utilized, processivity at this position was reduced by 36% and 28% by the C721A and M723A mutations, respectively (Table 1). With the heterologous TEL66 primer, the reduction in processivity was 54% for the C721A mutant and 51% for the M723A mutant (Table 1). In contrast, the W733A and H735A mutations resulted in little processivity defect (Table 1). Taken together, the in vitro and in vivo studies suggest that the E-i motif constitutes the primer grip in Est2p, and that this motif is a processivity determinant in both conventional RTs and TERTs.

Conservative Changes in RT Motifs of TERT Can Also Influence Telomere Length and Telomerase Processivity

Telomerase in vitro is distinguished from conventional RTs in having a low degree of processivity. Yeast telo-

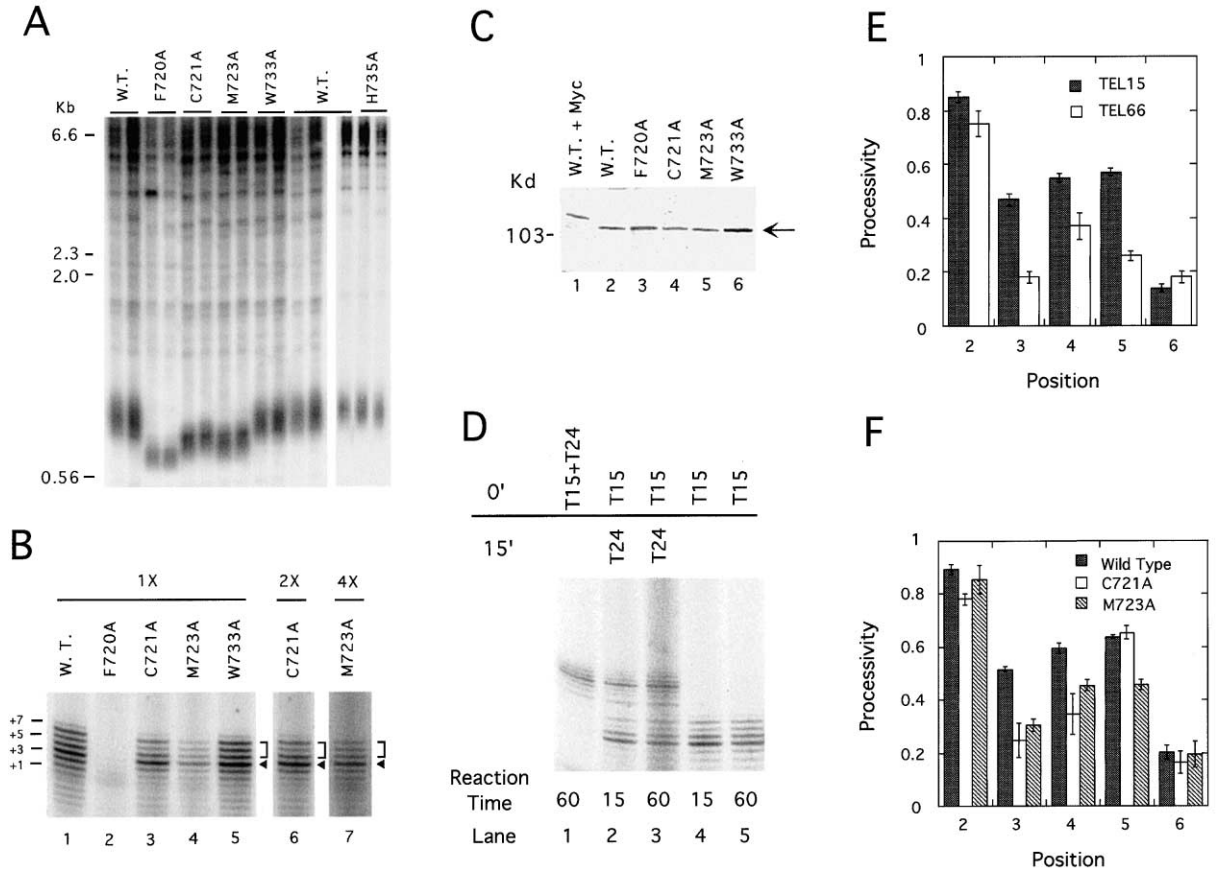

Figure 2. Functional Analysis of Potential Primer Grips in Est2p

(A) Telomere lengths were determined for strains bearing various mutated Est2ps. Two isolates of each mutant were tested.

(B) Telomerase from various mutant strains was isolated by IgG affinity chromatography and tested in primer extension assays using TEL15 as primer. To allow processivity differences to be more easily visualized, the C721A and M723A assays were exposed two and four times longer in lanes 6 and 7 such that the primer+3 bands (marked by a filled triangle) have comparable intensity to the band in the wild-type (W.T.) enzyme assay. The primer+4 to primer+6 bands (marked by brackets) for the C721A and M723A enzyme assays show reduced intensities, consistent with a processivity defect.

(C) Est2p protein levels in the wild-type and mutant strains were determined by Western blotting. The extract for lane 1 was prepared from a strain whose Est2p was fused to both a Myc_3 and a protein A tag. The reduced mobility of the immunoreactive species detected in lane 1 indicates that the assays were correctly detecting Est2p.

(D) The ability of telomerase to carry out processive DNA synthesis was tested by a competition assay. The enzyme was preincubated with 10 ng of TEL15 primer for 15 min (lanes 2–5), challenged with 300 ng of TEL24 primer (lanes 2 and 3), and immediately allowed to carry out polymerization by the addition of labeled and unlabeled nucleotide triphosphates. The reactions were terminated after an additional 15 min (lanes 2 and 4) or 60 min (lanes 3 and 5). For the reaction in lane 1, both TEL15 and TEL24 were added at the start of preincubation.

(E) Processivity of wild-type telomerase was determined using TEL15 or TEL66 as primer oligonucleotide. The reactions were performed in triplicates and the average processivity and standard deviation for the primer+2 to primer+6 positions plotted.

(F) Activities of wild-type and two mutant telomerases were tested using TEL15 as primer. Reactions were performed in triplicates, and the average processivity and standard deviation for the primer+2 to primer+6 positions were determined and plotted alongside the wild-type values.

merase is particularly inefficient in this regard, being capable of adding only a few nucleotides to the starting primer (Cohn and Blackburn, 1995; Lingner et al., 1997b; Lue and Peng, 1998; Prescott and Blackburn, 1997). Human and *Tetrahymena* telomerase, in contrast, can add hundreds or thousands of nucleotides to the starting primer (Greider, 1991; Morin, 1989). Conventional RTs likewise can polymerize thousands of nucleotides given the proper template and primer. Interestingly, the four amino acid residues located at the tip of the primer grip in HIV-1 RT and human TERT have the sequence "**W**-h-**G**-h" (h stands for hydrophobic or aliphatic residues), while the corresponding sequence in the E-i motif of Est2p is "**F**-C-**A**-M" (residues 720–723, Figure 1B). To determine if the "conservative" differences at position 1 and 3 of the motif can have significant effects on enzyme function, we made a double-substitution mutation converting the yeast motif to one that resembles more closely the human TERT and HIV-1 RT motif (FCA720WCG), and tested its effect on telomere maintenance and telomerase activity.

Remarkably, the strain bearing the FCA720WCG mutation exhibited normal growth and slightly longer telomere lengths in vivo (by 50–100 bps after 50 generations of growth; Figure 3A, compare lanes 7 and 8, and 9 and 10). In primer extension assays, overall DNA synthesis

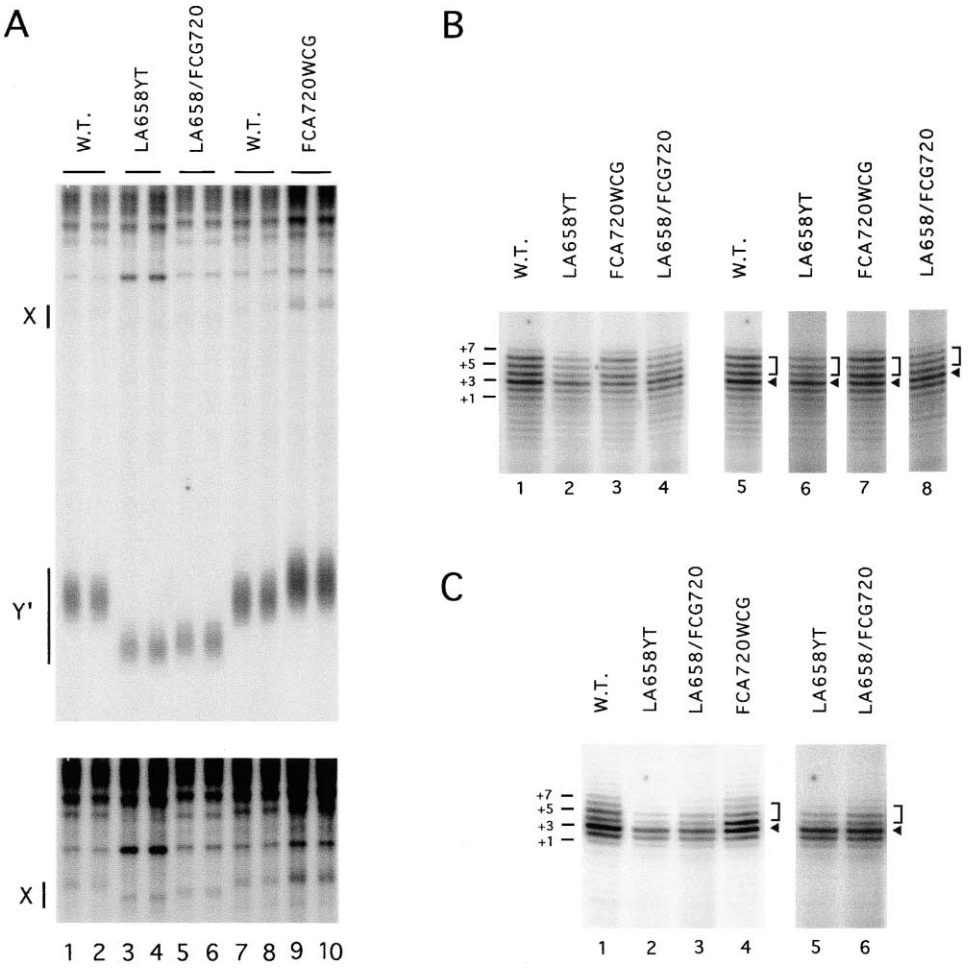

Figure 3. Functional Analysis of Motif C and Motif E Mutants of Est2p

(A) Telomere lengths were determined for strains bearing various mutated Est2ps. Two isolates of each mutant were tested. The top portion of the gel was exposed longer and shown at the bottom to allow better visualization of an X type telomere fragment. The locations of the Y′ type telomeres and an X type telomere are indicated by vertical bars.

(B) Telomerase from various mutant strains was isolated by IgG affinity chromatography and tested in primer extension assays using TEL15 as primer. To allow processivity differences to be more easily visualized, the assays for the mutants were exposed longer in lanes 6, 7, and 8 such that the primer+3 bands (marked by a filled triangle) have comparable intensity to the band in the wild-type (W.T.) enzyme assay (lane 5). Comparison of the intensities of the primer+4 to primer+6 bands (marked by brackets) for the wild-type and mutant enzymes gives an indication of processivity alteration. For example, the primer+4 and primer+6 band in the FCA720WCG assay have similar intensity as the primer+3 band (lane 7), while the same two bands in the wild-type assay have reduced intensity relative to the primer+3 band, suggesting that the FCA720WCG mutant has increased processivity.

(C) Telomerase from various mutant strains was tested in primer extension assays using TEL66 as primer. To allow processivity differences to be more easily visualized, the assays for two of the mutants were exposed longer in lanes 5 and 6 such that the primer+3 bands (marked by a filled triangle) have comparable intensity to the band in the wild-type (W.T.) enzyme assay (lane 1).

was slightly reduced by the mutation (Figure 3B, compare lanes 1 and 3; data not shown). However, careful determination of processivity revealed an increase of 24% and 26% at the primer+3 position for the TEL15 and TEL66 primers, respectively, suggesting that an increase in processivity is responsible for the increased telomere lengths (Figure 3B, compare lanes 5 and 7; Figure 3C, compare lanes 1 and 4; Table 1). Like the single-substitution primer grip mutants reported earlier, the double-mutant protein was present at nearly the wild-type level in the extract (data not shown).

Selection and characterization of drug-resistant mutants of HIV-1 RT have revealed a role for the conserved motif C in processivity control as well. This motif typically consists of two residues with hydrophobic or small polar side chains followed by two invariant aspartic acids (x_1-x_2-D-D), which are responsible for coordinating metal ions for polymerase chemistry. The identity of the two "x" residues varies among the cloned TERTs with x_1 being typically Phe or Leu and x_2 being Ala, Val, Thr, or Ile (Figure 1C) (Bryan et al., 1998; Malik et al., 2000). Conservative changes in the identity of the "x" residues have been found to influence HIV-1 RT processivity (Avidan and Hizi, 1998; Back et al., 1996; Caliendo et al., 1996). Recently, a mutated in vitro reconstituted *Tetrahymena* telomerase with Tyr-Thr for x_1-x_2 was found to exhibit greater processivity than similarly reconstituted wild-type enzyme (Bryan et al., 2000). To determine if

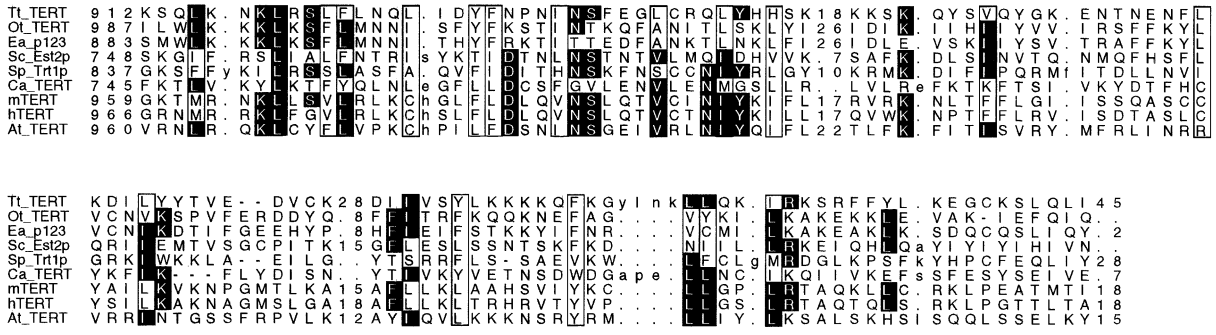

Figure 4. Alignment of the C-Terminal Extension of TERTs

An HMM alignment of the C-terminal extension of TERTs from nine species is shown. Well-conserved residues are shown in white (on black background) and hydrophobic residues shown in boxes.

the "x" residues affect yeast telomere length and telomerase function, we made a double-substitution mutant converting the x_1-x_2 of Est2p from L-A to Y-T (LA658YT), and tested its ability to support telomere maintenance and telomerase activity. Somewhat unexpectedly, the mutations resulted in greatly shortened telomeres and reduced growth (Figure 3A, lanes 1–4; Table 1). In vitro, overall DNA synthesis was reduced by ~20%, while a significant processivity defect can be observed (Figures 3B and 3C). For the TEL15 and TEL66 primer, the reduction in processivity at the primer+3 position was 39% and 56%, respectively (Table 1). Thus the same substitution in motif C can have opposite effects on Est2p and *Tetrahymena* TERT, suggesting that the x_1-x_2 residues work in concert with other residues in telomerase to promote processivity.

A quadruple mutant combining the LA658YT and FCA720WCG mutations was also generated and analyzed (LA658/FCA720). In this case, telomeres remain significantly shorter than those in wild-type cells, but slightly longer than those in the LA658YT strain (Figure 3A, lanes 1–6). The processivity of the quadruple mutant was found to be greater than the LA658YT mutant but still less than the wild-type enzyme (Figures 3B and 3C, Table 1), precisely as anticipated, if enzyme processivity is an important determinant of telomere length.

The C-Terminal Extension of TERT Contributes to Telomerase Processivity

In addition to the primer grip and conserved motif C, a region immediately C-terminal to the primer grip of HIV-1 RT, known as the thumb, (residues 237–317) has also been shown to contribute to enzyme processivity. Notably, all TERTs possess an ~150–200 amino acid region C-terminal to their respective primer grip. Though these C-terminal extensions (CTE) do not exhibit significant sequence similarity to the HIV-1 RT thumb domain, they appear to be loosely conserved among TERTs (Figure 4). In addition, secondary structure predictions consistently postulate a preponderance of α-helical structures in the CTE of TERTs (data not shown).

To determine if the CTE is functionally similar to the HIV-1 RT thumb domain, we generated four C-terminal deletion mutants, named C840, C800, C745, and C715; the number for each deletion mutant designates the number of the C-terminal most residues retained in the mutant. The C840 and C800 mutations remove part of the CTE; the C745 mutation removes all of the CTE, while the C715 mutation removes both the CTE and the primer grip of Est2p. Consistent with the critical role of the primer grip in reverse-transcriptase function, the C715 mutant failed to complement the growth defect of *EST2* disruption, and exhibited extremely short telomeres and undetectable telomerase activity (Figure 5A, lanes 7 and 8; Table 1). The three CTE deletion mutants also exhibited significantly reduced growth, although the C745 mutant was able to partially complement the growth defect. As expected, all three mutants possess greatly shortened telomeres (Figure 5A, lane 3–6; data not shown). Western analysis revealed significantly reduced Est2p levels for the C840 and C800 mutants and a normal level of Est2p for the C745 mutant (Figure 5B). This finding was corroborated by primer-extension studies that demonstrate greatly reduced overall DNA synthesis for the C840 and C800 mutants and a slight reduction for the C745 mutant (Figure 5C; data not shown).

Because of low-activity levels, the processivity of the C840 and C800 enzyme was not determined quantitatively. However, quantitation of the extension products from the C745 enzyme revealed a significant processivity defect, as evidenced from the underrepresentation of long products. For the TEL15 and TEL66 primer, processivity reduction of 44% and 74% for transcripts at the primer+3 position was observed (Figure 5C and Table 1). In contrast to the processivity defect, the total amount of DNA synthesis for the C745 enzyme was only slightly diminished, indicating that the CTE is not absolutely required for initiation of DNA synthesis.

To test the generality of the defect, the processivity of the wild-type and C745 enzymes was analyzed using two additional primers (TEL19 and TEL15-mt; Figure 5D). In each case, the C745 enzyme exhibited reduced processivity relative to the wild-type enzyme, as evident from the relative reduction in the amount of long-extension products (Figure 5D, lanes 1–4). To assess the role of nucleotide binding in the processivity change exhibited by the mutant, we performed telomerase assays using different combinations and concentrations of nucleotides. In standard assays, the labeled nucleotide dGTP was present at 0.2 μM, a limiting concentration.

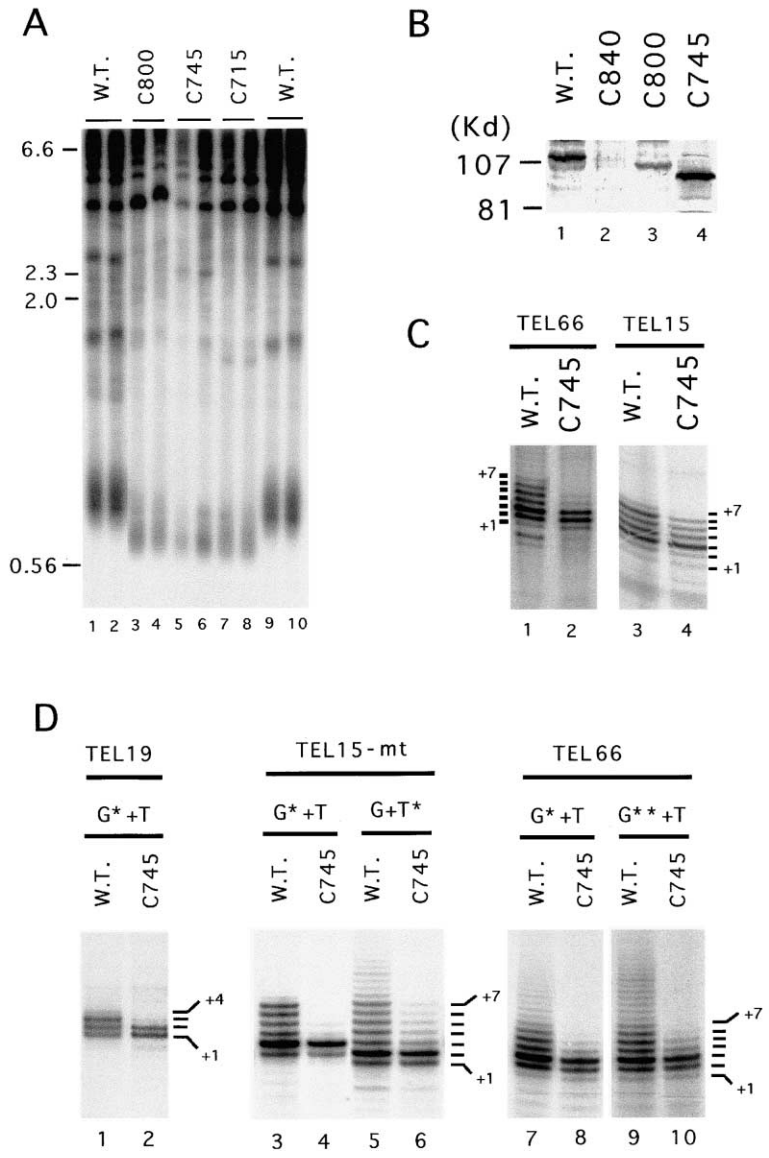

Figure 5. Functional Analysis of C-Terminal Truncation Mutants of Est2p

(A) Telomere lengths were determined for strains bearing various mutated Est2ps. Two isolates of each mutant were tested.

(B) Est2p protein levels in the wild-type and mutant strains were determined by Western blotting. The increased mobility of the reactive bands from strains with truncation mutants indicates that the assays were correctly detecting the protein A-tagged Est2p.

(C) Telomerase from various mutant strains was isolated by IgG affinity chromatography and tested in primer extension assays using TEL66 (lanes 1 and 2) or TEL15 (lanes 3 and 4) as primer.

(D) The wild-type (W.T.) and C745 enzyme were subjected to primer extension assays using the indicated primers and different combinations of labeled (indicated by * or **) and unlabeled nucleotide triphosphates. The unlabeled nucleotides were present at 50 μM, and the labeled nucleotides were present at either 0.2 μM (*) or 1.2 μM (**). The sizes of the labeled products relative to the starting primers are indicated to the right of each panel. The sequences of TEL19 and TEL15-mt are as follows: TEL19, TGTGTGACTGTGT GGGTGT; TEL15-mt, TGTGTGGTCACTGGG.

Changing the labeled nucleotide to dTTP significantly altered the banding pattern of the products (Figure 5D, compare lanes 3 and 5). Nevertheless, the C745 enzyme still exhibited a significantly reduced processivity (compare lanes 5 and 6). Raising the concentration of dGTP in standard assays from 0.2 to 1.2 μM (by adding unlabeled dGTP) increased the processivity of both the wild-type and C745 enzymes without abolishing the processivity defect of the latter (lanes 7–10). Similar results were obtained with the motif C and E mutants (data not shown). These data indicate that reduced nucleotide binding cannot account for the processivity defects of the telomerase mutants.

Comparison of the Catalytic Rate and Processivity of Telomerase Mutants with Telomere Maintenance

Nearly all of the TERT mutants exhibiting changes in processivity also manifest some generally minor alterations in catalytic activity as measured by total DNA synthesis (see Figures 2 and 3). To investigate if changes in catalytic rate of these mutants can account for changes in telomere length, we obtained kinetic values for the wild-type enzyme and selected TERT mutants. Two "processivity" mutants were chosen in this analysis, one causing a decrease in processivity and in telomere length (LA658YT), and the other causing an increase in both (FCA720WCG). For comparison, an N-terminal mutant that exhibits moderate telomere shortening but little change in enzyme processivity (IL290AA) was also tested in the kinetic assays. As shown in Table 2, and consistent with total activity assays (Figure 3), both the LA658YT and the FCA720WCG mutant exhibited an approximately 2-fold reduction in V_{max} relative to the wild-type enzyme. The N-terminal IL290AA mutant exhibited the lowest V_{max} (12-fold reduction), consistent with measurement of total DNA-synthesis activity (data not shown). Because the two processivitymutations have opposite effects on telomere length but nearly identical V_{max}, there is apparently a lack of correlation between catalytic rate and telomere length for these

Table 2. Comparison of Enzyme Properties and Telomere Maintenance for Selected Est2p Mutants

Mutation	K_m*	V_{max}*
None	1.1 (± 0.02)	20 (± 2.2)
LA658YT	0.52 (± 0.02)	9.8 (± 1.4)
FCA720WCG	0.59 (± 0.17)	9.8 (± 0.21)
IL290AA	0.95 (± 0.35)	1.7 (± 0.31)

*The K_m (in μM) and relative V_{max} values for each enzyme were determined in three independent experiments and the averages and standard deviations (in parentheses) reported.

mutants. That the IL290AA mutant exhibited the greatest reduction in V_{max} but the least reduction in telomere length also indicates that catalytic rate cannot be the sole determinant of telomere length. Inspection of the K_m values likewise reveals a lack of correlation between this parameter and telomere length. For example, the LA658YT and the FCA720WCG mutants have nearly identical K_m values for the telomeric primer, but have drastically different telomere lengths. Both of the processivity mutants also show apparently better binding (as evidenced by lower K_m) to the primer than the IL290AA mutant, yet one has longer and the other shorter telomeres in comparison. Thus, of all the enzymatic parameters examined, telomerase processivity appears to correlate best with telomere length.

Discussion

Distinct Elements of Telomerase Processivity

Synthesis of long extension products by telomerase depends upon two types of movement: simultaneous translocation of the RNA-DNA duplex away from the active site after each nucleotide incorporation (type I), and translocation of the RNP relative to the DNA primer after each cycle of copying the template (type II). Some telomerase (e.g., human and *Tetrahymena*) appears to be relatively proficient at both type I and type II translocations, thus capable of extending primers by hundreds of nucleotides. In contrast, yeast telomerase as isolated by standard procedures appears to be extremely inefficient at type I translocation, thus capable of completing at most one cycle of template copying (efficient type I translocation would have resulted in accumulation of the primer+7 product in standard assays). Because few of the transcripts actually reach the end of the template, it is difficult to determine quantitatively the efficiency of type II translocation for yeast telomerase. In this report, we have used the term processivity to refer exclusively to the ability of the enzyme to carry out type I translocation.

Implications of the Mechanistic Similarities Between HIV-1 RT and Yeast TERT

Similarity between the mechanisms of processivity control of HIV-1 RT and yeast TERT is evident from the mutational analysis. For both enzymes, processivity is governed, in part, by the conserved motif C, a primer grip-like motif (motif E), and a C-terminal extension (CTE). Because retroviral RTs and TERTs are evolutionarily distant, this finding suggests enzymes that are more closely related to the former (e.g., LTR retrotransposon RTs) or the latter (e.g., non-LTR element RTs and Group II Intron RTs) may also possess similar determinants of processivity (Eickbush, 1997; Nakamura and Cech, 1998). As two of the universally conserved RT motifs, motifs C and E should be capable of performing conserved functions. That the CTE of TERT can be shown to mediate optimal enzyme processivity raises the interesting possibility that it too may constitute a thumb for the polymerase.

Telomerase appears to be specifically activated in cancer cells and is believed to be a valid target for the development of anti-cancer therapeutics (Hahn et al., 1999; Herbert et al., 1999; Kim et al., 1994; Zhang et al., 1999). A strong motivation for targeting telomerase is the existence of clinically useful inhibitors of HIV-1 RT, a related enzyme. Such inhibitors fall into two major classes: (1) chain-terminating nucleoside analogs, which bind to the dNTP site, and (2) nonnucleoside RT inhibitors (NNRTI), which bind to a pocket nestled between the palm and thumb domain and lined in part by the primer grip (Huang et al., 1998). Our results showing the existence of the primer grip and the potential existence of a thumb domain in TERT thus suggest that both pockets may be available in telomerase and may be targeted by small-molecule inhibitors.

TERT: A Conserved Polypeptide with Distinct Functional Modules

Sequence and functional analysis to date suggests that the TERT protein may consist of at least three modules: an ~400 amino acid N-terminal extension, an ~300 amino acid central catalytic RT domain, and an ~150–200 amino acid C-terminal extension (CTE). As predicted, mutations in conserved residues in the central RT domain had detrimental effects on both telomere maintenance and telomerase activity (Haering et al., 2000; Harrington et al., 1997; Lingner et al., 1997b; Weinrich et al., 1997). Many of the analyzed mutations dramatically reduced total DNA synthesis in vitro, probably by affecting initiation. In this report, we demonstrate that besides affecting initiation, motif C and motif E in the RT domain can also have a profound effect on enzyme processivity. The CTE, on the other hand, appears to be primarily involved in supporting enzyme processivity. In contrast, the majority of mutations in the N-terminal extension appear to lead primarily to initiation defects (Friedman and Cech, 1999; Xia et al., 2000; Miller et al., 2000). Thus, the distinct modules of TERT may participate in distinct steps of the telomerase polymerization cycle.

A putative *C. elegans* homolog of TERTs (named CeTERT) was recently identified and was found to be missing conserved N-terminal motifs and the CTE (Malik et al., 2000). Our results on telomerase processivity suggest possible mechanisms for suppressing the defect associated with the lack of CTE. Because CTE appears to be primarily involved in processivity control, the CeTERT may have optimized other processivity determinants to compensate for the loss of the thumb domain. Consistent with the feasibility of such a mechanism, we have shown that incorporating the FCA720WCG mutation into the C745 enzyme can partially restore telo-

merase processivity and telomere length of the deletion mutant (data not shown).

Telomerase Processivity as a Limiting Determinant of Telomere Length

Telomere lengths in most organisms are maintained within a constant range, which is believed to be regulated by an interplay between the activity of telomerase and a set of protein factors that bind to telomeres and control the access of telomerase. With respect to the action of telomerase, earlier analysis argues convincingly that the nucleotide addition activity of telomerase is required for telomere maintenance (Lingner et al., 1997b). Results presented in this paper further suggest that the maintenance of physiologic telomere lengths is dependent upon the natural processivity of telomerase. As detailed earlier, among the mutations in motif C, the primer grip and CTE analyzed in this study, there is a good correlation between processivity alterations and telomere length alterations (Table 1). In contrast, kinetic analysis of selected mutants does not support a close correspondence between K_m and V_{max} on the one hand and telomere length on the other. Thus, at least among the processivity mutants, alterations in catalytic rate cannot by themselves account for alterations in telomere length. Furthermore, the FCA720WCG mutation, which reduces the catalytic rate of telomerase but increases enzyme processivity, caused an increase in telomere length, arguing for a dominant role of enzyme processivity in setting telomere length.

We cannot rule out the possibility that the processivity mutants may have unanticipated effects on other aspects of telomerase structure and function (e.g., interaction of Est2p with other telomerase components, interaction of telomerase anchor site and primer upstream region, the cleavage properties of telomerase, etc.). However, with regard to the other proven or putative telomerase components, deletion of either *EST1* or *EST3* has been reported to have no effect on telomerase activity (Hughes et al., 2000; Lingner et al., 1997a), whereas a loss of TLC1 interaction is expected to cause a defect in initiation. Thus, lack of association between Est2p and other telomerase components cannot by themselves explain the observed processivity changes in our mutants. Above all, the ability of telomerase with increased and decreased processivity to support the maintenance of increased and decreased telomere length argues strongly for an independent role of enzyme processivity in telomere length homeostasis.

Experimental Procedures

Yeast Strains and Plasmids

The construction of the Δest2 strain and that of pSE-Est2-proA has been described earlier (Xia et al., 2000). The Myc_3 tag located upstream of the protein A tag in pSE-Est2-proA was removed by inserting a PCR fragment encompassing residue 689–874 of Est2p in between the *Nco*I and *Xho*I site of the plasmid to give pSE-Est2-C874. This tagged and fully functional enzyme was referred throughout the text as the wild-type Est2p. For deletion mutants, fragments encompassing amino acids 689–840, 689–800, 689–745, and 689–715 of the *EST2* gene were amplified by PCR and inserted in between the *Nco*I and *Xho*I site of pSE-Est2-C874 to generate the pSE-Est2-C840, pSE-Est2-C800, pSE-Est2-C745, and pSE-Est2-C715 constructs, respectively. Thus, the number for each deletion mutant designates the number of the C-terminal most residue of Est2p retained in the mutant. All point mutations were generated by using the Quick-change protocol (Stratagene), appropriate primer oligonucleotides, and pSE-Est2-C874 as the template. All point mutations were confirmed by sequencing.

Determination of Telomere Length

To determine the effects of Est2p mutations on telomere maintenance, the pSE-Est2 plasmids carrying wild-type or mutated EST2 gene were transformed into an est2::Kan^R strain (either derived from KR36-6L or from W303-1a) that had been grown in the absence of Est2p for ~25–50 generations. Individual transformants were restreaked twice (50 generations), inoculated into 5 ml liquid medium, and grown to saturation. Chromosomal DNA was isolated and digested with *Pst* I restriction enzyme, and telomere lengths analyzed as before (Xia et al., 2000).

Extract Preparation and Assay for Yeast Telomerase.

Whole-cell extracts were prepared as previously described (Cohn and Blackburn, 1995; Lue and Peng, 1998; Lue and Xia, 1998). For affinity adsorption of telomerase, 4 mg of extract was mixed end-to-end with 20 μl of IgG-sepharose in binding buffer (10 mM Tris.HCl [pH 8.0], 1.2 mM magnesium chloride, 400 mM sodium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 0.05% Tween 20) at 4°C for 2 hr. The resin was then washed twice with 1 ml each of washing buffer (10 mM Tris.HCl [pH 8.0], 1.2 mM magnesium chloride, 600 mM sodium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol) and twice with 1 ml each of washing buffer without sodium acetate before the assay. Primer extension reaction is initiated by the addition of a 15 μl cocktail containing 100 mM Tris.HCl (pH 8.0), 4 mM magnesium chloride, 2 mM DTT, 2 mM spermidine, 100 μM dTTP, 10 μM primer oligodeoxynucleotides, and 20 μCi [α-32P]dGTP (3000 Ci/mmole). The reaction is allowed to proceed at 22°C for 1 hr, and the products recovered and analyzed as previously described (Lue and Peng, 1998). In some assays, a prelabeled 46-mer oligonucleotide that served as a recovery control was added to the reaction following primer extension and precipitated with the products.

For determination of processivity, the signal for each band was determined by Imagequant software and normalized to the amount of transcript by dividing against the number of labeled residues. Because most of the primers used end in 3 Gs, they can align to only one location along the yeast RNA template, allowing the sequence of the reaction products (TGTGGTG from +1 to +7) to be predicted precisely. The processivity for each position (P_i) was calculated using the formula $P_i = \text{sum}(T_{i+1} + T_{i+2} + \ldots + T_7)/\text{sum}(T_i + T_{i+1} + \ldots + T_7)$, where T_i designates the amount of transcript calculated for the primer+i position. Because P_3 appears to be most sensitive to mutations that alter processivity, the changes for P_3 (determined in triplicate assays) are selected for presentation in Table 1.

For determination of kinetic values, we carried out primer-extension reactions using IgG-Sepharose purified enzyme, varying concentrations of a 15 nt primer (TGTGTGGTGTCTGGG), and 25 μCi [α-32P]dTTP (3000 Ci/mmole). This combination of enzyme and substrates allow the addition of only one nucleotide to the starting primer, thus eliminating the confounding effect of enzyme processivity on product accumulation. The reactions were stopped after 6 min, a time point within the linear range of DNA synthesis. The K_m (for the primer) and V_{max} values were determined by obtaining least square fitting lines of the data in Edie-Hofstee plots and then calculating the negative slope and y intercept, respectively. Values for each enzyme were obtained from three independent experiments, and the averages and standard deviations computed.

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