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 RT's encoded by retroviruses and retroelements. The polymerization mechanisms of telomerase may therefore be similar to those of the “conventional” RT's. 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 RT's 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 RT's and underscores the roles of distinct aspects of telomerase biochemistry in telomere length maintenance.

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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, first purified from Euplotes aediculatus as p123, was found to be homologous to Est2p, a yeast protein required for telomere maintenance (7–9). Both the rabbit reticulocyte lysate system suffices to reconstitute enzyme activity (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 RT's 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 RT's 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 RT's may possess very similar polymerization mechanisms.

We have sought to clarify the extent of mechanistic conservation between TERT and conventional RT's 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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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 Δest2 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 Quick Change protocol (Stratagene), appropriate primer oligonucleotides, and pSE-Est2-C874 as template. All point mutations were confirmed by sequencing.

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

**Purification 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 μg 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 (TGGGAG). The processivity for each position (*P*) was calculated using the following formula:

$$ P = \frac{\sum(T_{i+1} + T_{i+2} + \ldots + T_n) \times \sum(T_{i+1} + T_{i+2} + \ldots + T_n)}{n} $$

where *T* 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 deproteinized 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 BamHI and EcoRV sites of pBluescript II KS±. The resulting plasmid was linearized by digestion with *Hind*III, and antisense RNA encompassing residues 1097–1301 of the TLC1 gene was generated by T3 RNA polymerase in the presence of 12 μM [α-32P]GTP as described (36).

**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. 1A). In atomic resolution structural models of HIV-1 RT, motifs 1 and 2 constitute a long β-hairpin, the base of...
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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. 2B, 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. 2C, 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 (TGGTGGTGT-GTGGG), which consists of canonical yeast telomere repeats, and TEL66 (TAGGGTAGTAGGGG), 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. 4A). 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 Lys443 and Arg450 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%...
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), and with the notion that telomerase may exhibit conformational heterogeneity as its

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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. 6A) (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. 6A). 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. 6B). 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).
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 (Lys443 and Arg450); residues tolerant of conservative substitutions are less important (Ile441 and Ile452); 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 substrate 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 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.

Comparison of the Biochemical Defects of TERT and HIV-1 RT Mutants with Amino Acid Substitutions in the Finger Domain—Because both Lys443 and Arg450 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 (Lys65 and Arg72) 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 Lys65 on DNA synthesis and processivity appears to be much milder than that at Arg72 (44).

Alanine substitution of Ile452 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 (Leu74) in HIV-1 RT. In

![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 dGTP is plotted. B, the processivity of wild-type and mutant telomerase at the P+2 position in the presence of increasing concentrations of labeled dGTP are plotted. C, 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.](http://www.jbc.org/)
the covalently trapped catalytic complex of HIV-1 RT, Leu74 appears to “lock the templating base tightly in place” (Fig. 1B) (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 Lys443, Arg450, and Ile452, the residues in HIV-1 RT that correspond to Lys437 (possibly Pro439) and Ile441 (Ile463) 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 Lys437, Lys443, and Arg450 of Est2p have all been mutated and tested in this in vitro system (Lys532, Lys538, and Arg543 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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