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 (RT),1 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 from a wide range of organisms and genes encoding both the RNA and protein components of the enzyme complex identified (for reviews see (3, 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 proteins possess RT-like motifs, alterations therein can cause inactivation of telomerase activity and reduction in telomere lengths. Subsequently, homologs of TERT were identified in Schizosaccharomyces pombe, human, mouse, Tetrahymena, Oxytricha, and Arabidopsis (10–17). Because co-expression of TERT and telomerase RNA in vitro in the rabbit reticuloocyte lysate system suffices to reconstitute enzyme activity (18, 19), 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 (20–24).

Extensive 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 analogues of RT residues essential for catalysis are absolutely required for telomerase activity and telomere maintenance (9, 18, 25–27). Conserved residues shown previously to modulate RT processivity have been found to be important determinants of telomerase processivity as well (28–30). In addition, the same tyrosine residue in conserved motif A allows both TERT and RTs to discriminate against incorporating ribonucleotides (31). However, some other crucial RT residues (e.g. a Gln in motif B’) appear to be less important or even dispensable for telomerase function (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.

In retroviral RTs, the region immediately C-terminal to the RT motifs (known as the "thumb" domain) has been shown to be important in template/primer binding and in processive nucleotide addition. Such functions are readily comprehended in light of the structural disposition of the thumb domain, which comprises a bundle of three α-helices, part of which makes direct contact with the template/primer duplex (32). The comparable region of TERTs (henceforth referred to as the CTE for C-terminal extension) exhibits no evident sequence homology to retroviral RTs. However, previous work from our laboratory indicates that the CTE of yeast TERT (Est2p) is also required for optimal enzyme processivity, raising the possibil-
ity that the CTE may also constitute a thumb for telomerase. In this report, we explored the mechanistic basis for the function of telomerase CTE and found that it enhances the stability of telomerase-DNA interaction both before and after polymerization. The CTE of Est2p also possesses a nucleic acid binding activity when recombimantly expressed and purified. Finally, the CTEs of TERTs as a group appear to exhibit a low level of sequence conservation, and substitution of quite a few conserved CTE residues in Est2p was found to impair telomerase activity and processivity. Taken together, our study supports the notion that the CTE of TERT has a conserved function in promoting primer binding and telomerase processivity and that mechanistic similarity between telomerase and conventional RTs may be greater than indicated by apparent sequence similarity.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Primer DNA—The construction of a Δest2 strain harboring the pSe-Est2-C874 plasmid (containing a protein A-tagged EST2 gene) has been described (33). This fully functional Est2p is designated as wild type telomerase throughout the text. The construction of the protein A-tagged C745 mutant has been described also (29). All substitution mutations in the CTE of EST2 were generated using the QuikChange protocol (Stratagene), appropriate primer oligonucleotides, and pSe-Est2-C874 as template. All point mutations were confirmed by sequencing. The oligodeoxynucleotide primers used for telomerase assays were purchased from Sigma and purified by denaturing gel electrophoresis prior to use. The primers have the following sequences: TEL15(m12), TGGTCTGCTGCTGGG; TEL24, TCTGCGG-GTTGCTGGTTGCTGGG; TEL66, TAGGTAAGTAAGGGG.

Purification of and Assay for Yeast Telomerase—Whole cell extracts and IgG-Sepharose-purified telomerase were prepared as described previously (29, 33–35). 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, 1 mM dithiothreitol, 2 mM spermidine, primer oligodeoxynucleotides, and varying combinations of labeled and unlabeled dGTP and dTTP. Primer extension products were processed and analyzed by gel electrophoresis as described previously (35, 36).

For the primer challenge experiment, the IgG-Sepharose-bound telomerase was preincubated with 40 ng of TEL15(m12) primer at room temperature for 15 min and then challenged with 100-fold excess of a test primer. After 5, 15, and 40 min, labeled nucleotides (dTTP, 25 μCi) were added to initiate the polymerization reaction. For the measurement of enzyme-product stability, reactions were performed as described previously. Upon termination, the IgG-Sepharose was washed three times with 100 μl each of TMG-10 (150) buffer. The washes were pooled, digested with proteinase K, and extracted with phenol/chloroform. The remaining nucleic acids were combined with an antisense probe (100,000 cpm) and hybridized and digested as described previously (39). For synthesis of uniformly labeled RNA probe, the TLC1 gene (nucleotides 1–1301) (40) was first amplified by PCR and cloned in between the BamHI and EcoRV site of pBluescript II KS+. The resulting plasmid was linearized by digestion with HindIII, 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 (39). The levels of Est2p in cell extracts were determined by Western analysis as described previously (30).

RESULTS

Removal of the C-terminal Extension of TERT Causes a Reduction in the Stability of Telomerase-DNA Interaction—To probe the function of the CTE, we generated a deletion mutant of Est2p (named C745) that removed most of the amino acids beyond the last conserved RT motif (Fig. 1A, motif E). A yeast strain harboring this deletion mutant was severely defective in telomere maintenance, and telomerase isolated from the strain exhibits a severe reduction in enzyme processivity (29). To further analyze the enzymatic defects associated with the removal of CTE, we measured the stability of telomerase-DNA binding using a primer-challenge assay. A test primer (TEL15(m12)) was pre-bound to either wild type or C745-containing telomerase followed by the addition of an excess of a competitor primer (TEL24). A labeled nucleotide was then added after various time intervals to initiate the extension reaction. The amounts of reaction products arising from the test primer were then quantified. As shown in Fig. 1, B and C, either reverse phase HPLC or gel electrophoresis on the test primer more quickly when the C745 enzyme was used in the assays. Inspection of Fig. 1C indicates that the half-life of the telomerase-DNA complex for the TEL15(m12) primer was reduced by −2-fold by the C-terminal truncation mutation (from about 2 to 1 min). These results are consistent with a role for CTE in telomerase-DNA interaction.
Fig. 1. The C-terminal extension of Est2p enhances the stability of telomerase-DNA interaction. A, a schematic illustration of the structure of Est2p showing the locations of the conserved RT motifs, the N-terminal extension, and the C-terminal extension. B, wild type and C745 telomerase were subjected to the primer challenge protocol as described under “Materials and Methods.” Products arising from extension of either TEL24 or TEL15(m12) are indicated by brackets to the left of the panel. The time intervals (in min) between the addition of the TEL24 primer and the nucleotides are indicated at the top. C, the signals for the test primer (TEL15(m12)) were normalized against the signal obtained in the absence of the challenge primer (TEL24) and plotted as a function of time.

We also measured the stability of telomerase-DNA binding after the primer has been extended. For this analysis, we took advantage of the stable association between protein A-tagged telomerase and IgG-Sepharose. Reactions were performed in duplicates using the TEL15(m12) primer. Upon termination of the reactions, the labeled products were separated into soluble and Sepharose-bound fractions before analysis in denaturing gels (Fig. 2A). The ratios of enzyme-bound products to free products at each extension position were then quantified. For ease of presentation, only the results for the Primer + 3 (p + 3) and Primer + 5 (p + 5) position are shown in Fig. 2B. At both positions, the C745 enzyme bound a smaller fraction of the reaction products than the wild type enzyme, consistent with impaired telomerase-DNA interaction. Similar results were obtained at other positions. It was also evident from the analysis that for both the wild type and C745 enzyme, the fraction of enzyme-bound products was higher at the p + 5 than at the p + 3 position, consistent with the ability of a longer RNA-DNA hybrid to promote enzyme-DNA stability.

As yet another measure of telomerase-DNA interaction, we carried out kinetic analysis of nucleotide addition for both the wild type and mutant enzyme. Assays were performed using varying concentrations of a primer oligonucleotide, and the results were analyzed by Eadie-Hofstee plots. As shown in Fig. 3, A and B, the $K_m$ for the C745 telomerase was $2.5$-fold higher than that for the wild type enzyme, consistent with reduced binding of the primer to the truncated telomerase.

Thus, by three different criteria, the loss of the CTE of yeast TERT resulted in a telomerase that binds less strongly to telomeric DNA.

The C-terminal Extension of TERT Can Bind Nucleic Acids—To determine whether the CTE of TERT can indeed bind nucleic acids as would be expected for the thumb domain, we expressed amino acids 747–874 of yeast TERT as a fusion protein to an intein-CBD (chitin-binding domain) tag. The fusion protein was purified by adsorption to a chitin column, and the CTE was separated from the tag by intein-mediated self-cleavage in the presence of dithiothreitol. For unknown reasons, the CTE fragment remained bound to the column after cleavage and could not be eluted even with high salt. We therefore eluted the CTE fragment with urea. Following elution, the CTE was purified further either with a reverse phase HPLC or an S-Sepharose column. The HPLC preparation was lyophilized and solubilized in water, whereas the S-Sepharose preparation was renatured by stepwise dialysis into buffers containing reducing concentrations of urea. As shown in Fig. 4A, both preparations are nearly homogeneous. We then subjected the HPLC-purified fragment to analytical ultracentrifugation and CD analysis. These studies revealed a monomeric protein with high $\alpha$-helical content (data not shown). Thus, despite the denaturation step during isolation, the purified CTE appears to be well folded.

The purified CTE was tested for its ability to interact with nucleic acids using a nitrocellulose filter-binding assay. First, a single-stranded 24-mer oligonucleotide with a telomere-like sequence was assessed for binding. As shown in Fig. 4B, the S-Sepharose-purified CTE exhibited concentration-dependent
binding to this oligonucleotide, whereas a control protein (maltose-binding protein) did not. We then compared the ability of single-stranded DNA, double-stranded DNA, and RNA-DNA hybrid (all with telomere-like sequences) to bind CTE. Equal molar concentrations of the different substrates were tested in filter-binding assays using the same amount of CTE. Interestingly, as shown in Fig. 4C, CTE appears to manifest a slight preference for double-stranded substrates (~2-3-fold), consistent with its putative role in contacting an RNA-DNA hybrid in the context of a telomerase-DNA complex. Analysis of nucleic acid binding by the HPLC-purified CTE gave comparable results (data not shown).

**Conserved Residues in the CTE of Yeast TERT Are Required for Telomerase Activity and Processivity**—Even though the CTEs of TERTs do not exhibit significant sequence similarity to retroviral RTs, they appear to be loosely conserved as a group (29). To determine whether the sequence conservation has functional significance, we mutated conserved residues within the CTE of yeast TERT and tested the resulting enzyme for defects both in vivo and in vitro. A total of five mutants with substitutions in conserved residues (SCR mutants) were made, each with two or three consecutive residues changed to alanines: LF759AA, TID768AAA, NS774AA, YK794AA, FL847AA (with the number designating the position of the first amino acid residue in each pair or triplet; Fig. 5A). For comparison, a mutant with substitutions in non-conserved residues was also made (CD831AA). Each mutant was tagged at the C terminus with tandem copies of the IgG-binding domain of proteins A, placed on a centromeric plasmid, and used to transform a yeast strain whose chromosomal EST2 (TERT) gene has been disrupted. A similarly tagged wild type EST2 gene was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. B, and the CTE (purified by an S column and Maltose-binding protein (MBP)) were tested for binding to 1 nM of three different substrates. The single strand DNA is a 15-mer oligonucleotide with a telomere-like sequence (TGTGTGGTGGTGTGTGGG), and the results were plotted. C, the CTE (purified by an S column, 4 μg) was tested for binding to 1 nM of three different substrates. The single strand DNA is a 15-mer oligonucleotide with a telomere-like sequence (TGTGTGGTGGTGTGTGGG). The double strand DNA and RNA-DNA hybrid were generated by annealing the same oligonucleotide to fully complementary DNA and RNA oligomers, respectively. The background was subtracted from the signals and the results plotted. NS774AA, and YK794AA) have telomeres that are indistinguishable from the C745 truncation mutant, suggesting that these substitutions have completely abolished the function of CTE in vivo.

To determine whether the telomere maintenance defects can be explained by telomerase activity loss or alteration, we purified protein A-tagged telomerase from the wild type and mutant strains by IgG-Sepharose and subjected it to primer extension assays (Fig. 6A). The results were analyzed both in terms of the overall levels of DNA synthesis (Fig. 6B) and the processivity of nucleotide addition (Fig. 6C). As expected, the CD831AA mutant exhibited a nearly normal level of telomerase activity and a normal pattern of extension, consistent with its ability to maintain normal telomere lengths. In contrast, all of the SRC mutants suffered either a loss of total activity, a significant reduction in enzyme processivity, or both. Interestingly, the two mutants with total activity loss but no evident processivity defect (LF759AA and FL847AA) manifested relatively modest telomere shortening such that the levels of activity correlated with the lengths of telomeres. On the other hand, the three mutants with substantial processivity defects (TID768AAA, NS774AA, and YK794AA) all manifested severe telomere shortening. Of the three, only the YK794AA mutant suffered substantial loss of total activity (>10-fold). These results are in accordance with earlier studies and indicate that both the total activity and the processivity of telomerase are important determinants of telomere lengths. Processivity appears to be the more dominant factor, because only a modest reduction in processivity was sufficient to trigger severe telomere shortening (29).
The substantial loss of overall activity exhibited by the YK794AA and the FL847AA mutants are surprising in light of previous analysis of the C745 truncation mutant, which has no CTE yet suffers only a modest reduction in overall activity. To determine the basis for the activity loss manifested by the substitutions mutants, we quantified the levels of Est2p and Est2p-associated TLC1 RNA (yeast telomerase RNA) in wild type and mutant strains. As shown in Fig. 7, A and B, both the YK794AA and FL847AA mutants have much lower levels of Est2p and Est2p-associated TLC1 RNA in cell extracts, whereas the other mutants have very few defects in this regard. Quantitation indicates that the magnitude of reduction for both RNA and protein was ∼4–10-fold (data not shown). These results suggest that substitution in some conserved CTE residues can impair protein stability (possibly by causing protein misfolding), thereby leading to a reduction in the level of telomerase ribonucleoprotein.

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DISCUSSION

The Function of the C-terminal Extension of Telomerase Reverse Transcriptase—We have shown in this and an earlier study (29) that the CTE of yeast TERT is required for optimal telomerase processivity and stable telomerase-DNA binding. The yeast CTE also possesses a nucleic acid binding activity when recombinantly overexpressed and purified. These results support the notion that despite the lack of sequence similarity between CTE and the thumb domain of conventional RTs, the CTE of TERT may nonetheless act as a thumb for telomerase.

A precedent for sequence divergence coupled with functional (and potential structural) similarity was noted in structural
Telomerase from several species has been shown to form dimers-multimers in vitro and in vivo, although the subunit or domain of the complex responsible for dimerization is not well characterized. The functional significance of dimerization also remains to be determined. The monomeric behavior of the yeast CTE can have similar deleterious effects. Second, the nucleic acid binding function of the CTE may be more or less essential for telomerase activity, depending on the presence of other nucleic acid binding modules/domains in the enzyme complex. For example, the yeast telomerase reconstituted in vivo may possess other nucleic acid binding components that can partially compensate for the loss of CTE. In this regard, it is interesting to note that Est1p, a non-catalytic component of yeast telomerase, has been shown to possess a single-stranded telomeric DNA binding activity (47, 48). Another distinguishing feature of the yeast enzyme is its relatively long RNA template (17 nucleotides instead of 9 or 10 nucleotides for the Tetrahymena and human RNA), which can presumably bind more strongly to the DNA primer and possibly alleviate the consequence of CTE loss.

Although our evidence favors at least one conserved function for CTE, it by no means rules out some additional species-specific functions such as interaction with other components of the telomerase holoenzyme. In particular, the addition of an epitope tag to the C terminus of hTERT resulted in a catalytically active telomerase that is unable to maintain telomeres in vivo, a finding that argues for just such a species-specific function (49).

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