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**DNA: REPLICATION REPAIR AND  
RECOMBINATION:  
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# Conserved N-terminal Motifs of Telomerase Reverse Transcriptase Required for Ribonucleoprotein Assembly *in Vivo*\*

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Telomerase is a ribonucleoprotein (RNP) reverse transcriptase responsible for the maintenance of one strand of the telomere terminal repeats. The key protein subunit of the telomerase complex, known as TERT, possesses reverse transcriptase (RT)-like motifs that directly mediate nucleotide addition. The RT motifs are located in the C-terminal region of the polypeptide. Sequence alignments also revealed the existence of four conserved motifs (named GQ, CP, QFP, and T) in the N-terminal region of TERT. The GQ motif of yeast TERT has been demonstrated previously to be essential for telomerase catalysis and may participate in RNP formation. In this report, we show that substitution of conserved residues in the CP, QFP, and T motifs of yeast TERT also impairs both telomere maintenance and telomerase activity, thus confirming the validity of the sequence alignment. The extent of telomere shortening correlates with the extent of reduction in the level of telomerase activity, TERT protein, and TERT-associated TLC1 RNA. Overexpression of the mutant proteins does not result in telomere shortening, implying that assembly rather than catalytic function was affected. This notion was further supported by comparing the efficiency of RNP formation in the wild type and the overexpression strains. Taken together, our results show that three of the four N-terminal motifs are required for efficient telomerase RNP formation *in vivo* but not for the enzymatic function of telomerase. We also show that the majority of telomerase-associated TLC1 RNA has a more upstream 3' end than previously reported, consistent with additional processing events during RNP maturation.

Telomerase is a ribonucleoprotein (RNP)<sup>1</sup> 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 dG-rich strand of telomeres (2).

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<sup>1</sup> The abbreviations used are: RNP, ribonucleoprotein; RT, reverse transcriptase; TERTs, telomerase reverse transcriptases; nt, nucleotide.

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 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 protein from *Saccharomyces cerevisiae* required for telomere maintenance (7–9). Both proteins possess reverse transcriptase (RT)-like motifs, alterations in which render telomerase inactive both *in vitro* and *in vivo*. Subsequently, homologues of TERT were identified in a phylogenetically diverse group of organisms (10–17). Because co-expression of TERT and telomerase RNA in rabbit reticulocyte lysates suffices to reconstitute enzyme activity (18, 19), these two subunits probably constitute the core of the enzyme complex. Quite a few telomerase-associated polypeptides have been identified using either biochemical or genetic tools. Studies from several laboratories (20–24) suggest that these factors may participate in telomerase assembly, catalytic function, or regulation.

The RT-like motifs are located in the C-terminal region of cloned TERTs. Extensive mutational analysis of these motifs in TERTs supports an overall conservation of basic catalytic mechanisms between telomerase and conventional RTs. 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 (28–30) to modulate RT processivity have been found to be important determinants of telomerase processivity as well. In addition, the same tyrosine residue in conserved motif A allows both TERTs 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 catalysis (9). Taken together, these results suggest that despite the high degree of sequence divergence (<20% sequence identity), the RT domains in both classes of proteins are mechanistically quite similar.

Detailed sequence analysis of nine cloned TERTs revealed, in addition to the RT motifs, four telomerase-specific motifs (named GQ, CP, QFP, and T) positioned N-terminal to the RT region (32). The functions of selected TERT N-terminal residues have been analyzed by reconstituting mutated TERT protein with telomerase RNA either *in vitro* (*Tetrahymena* and human) or *in vivo* (*S. cerevisiae*, *Schizosaccharomyces pombe*, and human) (18, 27, 31–33). In addition to confirming the functional importance of many of the conserved residues, the

results from *Tetrahymena* indicate that the CP and T motifs may be required for TERT binding to telomerase RNA *in vitro* (33, 34).

We have initiated a detailed structure-function analysis of the N-terminal region of *S. cerevisiae* TERT (Est2p). In an earlier report (32), we showed that the most N-terminal GQ motif is required for telomere maintenance and, in some cases, for telomerase activity *in vitro*. Overexpression of some of the mutants resulted in telomere shortening, implying that telomerase catalytic function, rather than RNP formation, was impaired by the mutations. We now report an analysis of the remaining three N-terminal motifs using an extensive panel of mutations. Our results confirm the functional importance of the CP, QFP, and T motifs in both telomere maintenance and telomerase activity. In addition, for mutations in these motifs, the extent of telomerase activity loss correlates with the extent of reduction in the amount of TERT protein and TERT-associated TLC1 RNA. Overexpression of the mutant proteins does not result in telomere shortening, implying that assembly rather than catalytic function was affected. This notion was further supported by a comparison between the efficiency of RNP formation in the wild type and the overexpression strains. Taken together, our results imply that three of the four N-terminal motifs in TERTs are required for efficient RNP formation *in vivo* but not for the telomere extension function of telomerase. We also show that the majority of telomerase-associated TLC1 RNA has a more upstream 3' end than reported previously, consistent with additional processing events during RNP maturation.

#### MATERIALS AND METHODS

**Yeast Strains and Plasmids**—The construction of an *est2-Δ* strain harboring the pSE-Est2-C874 plasmid (containing a protein A-tagged *EST2* gene) has been described (32). This fully functional Est2p is designated wild type telomerase throughout the text. The construction of the protein A-tagged C745 mutant has also been described (29). All substitution mutations in the CP, QFP, and T motifs of *EST2* were generated by using the Quick-Change protocol (Stratagene), appropriate primer oligonucleotides, and pSE-Est2-C874 as template. All point mutations were confirmed by sequencing. The oligodeoxynucleotide primers used for mutagenesis were purchased from Sigma-Genosys and purified by denaturing gel electrophoresis prior to use. For overexpression of TERT mutants, a vector containing the triose-phosphate isomerase promoter (pYX232, Ingenious Inc.) was utilized. The *NcoI* site within the polylinker of pYX232 was converted to an *NdeI* site, and the *NdeI-SalI* fragments from the pSE-Est2-C874 series of plasmids were inserted between the *NdeI* and *SalI* site of the resulting vector (32).

**Comparative Sequence Analysis**—All sequences used in comparative analysis were obtained from NCBI website at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov). The final alignment was based on manual adjustment of a hidden Markov model as described previously (32).

**Analysis of Telomere Length**—The *est2-Δ* and W303 strain were transformed with the pSE and pYX series of plasmids, respectively. Independent clones were re-streaked twice on plates. Chromosomal DNAs were then isolated using the "Smash and Grab" protocol, digested with *PstI*, and electrophoretically separated on a 0.9% agarose gel. Following capillary transfer to nylon membranes, telomere-containing fragments were detected by hybridization with a <sup>32</sup>P-labeled poly(dG-dT) probe (32).

**Purification of and Assay for Yeast Telomerase**—Whole cell extracts, DEAE fractions, and IgG-Sepharose-purified telomerase were prepared as described previously (29, 32, 35, 36). Each primer extension assay was carried out using 20 μl of IgG-Sepharose pretreated with 4 mg of protein extract and was initiated by the addition of a 15-μl mixture containing 100 mM Tris-HCl, pH 8.0, 4 mM magnesium chloride, 2 mM dithiothreitol, 2 mM spermidine, 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 (36, 37). The oligodeoxynucleotide primers used for telomerase assays were purchased from Sigma-Genosys and purified by denaturing gel electrophoresis prior to use. The primers have the following sequences: TEL15, TGTGTGGTGTGTGGG; TEL66, TAG-GGTAGTAGTAGGG.

**Protein and RNA Analysis**—The levels of protein A-tagged yeast TERT in cells extracts were determined by Western blotting as described previously (30). RNase protection studies were carried out as follows. A PCR fragment that spans nucleotide 1–1301 of the *TLC1* gene (38) was generated and cloned between the *BamHI* and *EcoRV* site of pBluescript II KS+ to give pBS-TLC1. For the synthesis of labeled antisense probe, pBS-TLC1 was linearized by digestion with *HinfI* and transcribed with T3 RNA polymerase in the presence of 12 μM [ $\alpha$ -<sup>32</sup>P]GTP as described (39). For the synthesis of the 1–1300 and 1–917 sense transcripts, the same plasmid was digested with *XhoI* and *AflII*, respectively, and transcribed with T7 RNA polymerase. For the protection assay, total RNAs from DEAE fractions or *in vitro* transcription reactions were combined with the probe (100,000 cpm), and the mixtures were precipitated with ethanol. The RNAs were then hybridized in 80% formamide, digested successively with RNase T1, RNase A, and proteinase K, and analyzed with gel electrophoresis as described (40).

**High Resolution Mapping of the TLC1 RNA 3' End**—Total RNAs from DEAE fractions or from *in vitro* transcription reactions were isolated by proteinase K digestion, phenol/chloroform/isoamyl alcohol extraction, and ethanol precipitation. The RNAs were then polyadenylated using yeast poly(A) polymerase and reverse transcribed using a poly(dT)-containing primer (CCGGAATTCCTTTTTTTTTTTTTTTTTT) (41). cDNAs spanning the 3' end of TLC1 RNA were amplified by PCR using a TLC1-internal primer (CGGGATCCGATCAGTAACTGAACAA-TGAC) and the same poly(dT) primer. Following gel purification and restriction enzyme cleavage, the fragments were cloned between the *EcoRI* and *BamHI* site of pBluescript II KS+, and the TLC1-poly(A) junctions were determined by sequencing.

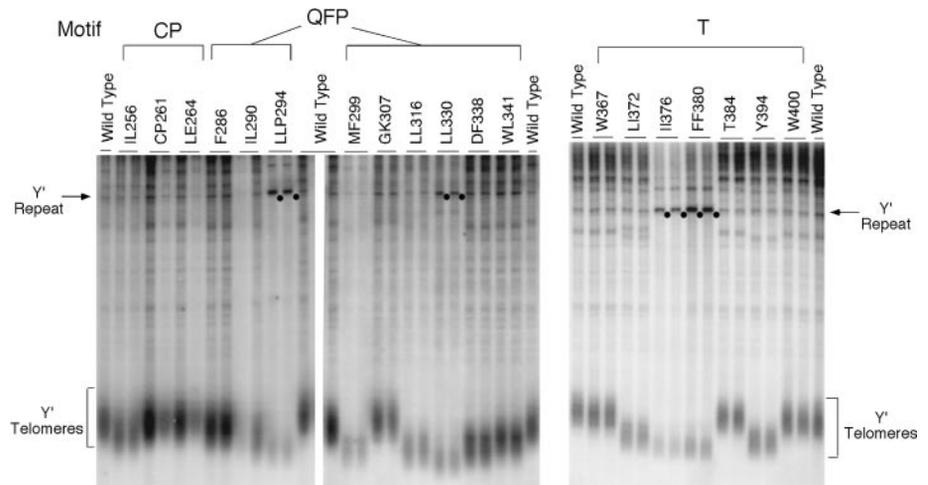
#### RESULTS

**Previously Identified N-terminal Motifs of TERT Are Conserved in Newly Discovered Homologues**—Through the use of a hidden Markov model, we had earlier reported the identification of four conserved motifs in the N-terminal extension of TERTs from ciliates, fungi, mammals, and a plant (32). Subsequently, sequences for 11 additional TERTs were reported, including those from *Cryptosporidium parvum*, *Encephalitozoon cuniculi*, *Giardia lamblia*, *Mesocricetus auratus*, *Moneoplothes crassus*, *Oryza sativa*, *Paramecium caudatum*, *Plasmodium yoelii*, *Plasmodium falciparum*, *Xenopus laevis*, and *Caenorhabditis elegans* (42–50). Some of the newly discovered homologues manifest a surprising degree of evolutionary divergence. For example, the TERTs from *P. falciparum* and *P. yoelii* are more than twice the size of many other proteins of this family. Nevertheless, a realignment of the N-terminal extension suggests that, with the exception of CeTERT, the four N-terminal motifs are universally conserved (Fig. 1). Close inspection of the new alignment revealed the following two interesting features. First, even though there are very few absolutely conserved residues, many positions appear to tolerate only conservative substitutions. A great majority of these conserved positions favor hydrophobic or aromatic residues, which may correspond to key positions in the core of a domain or surface locations crucial for interactions with other domains or molecules. Second, the proposed linker region between the GQ and CP motif is even more variable in length than previously suspected, ranging in size from 19 residues to >500. Interestingly, the smallest TERT (*E. cuniculi*) also possesses the smallest linker.

**The CP, QFP, and T Motifs of Yeast TERT Are Required for Telomere Maintenance and Telomerase Enzyme Activity**—To extend our earlier functional analysis of the GQ motif, we created a series of substitution mutants of yeast TERT with alterations in the CP, QFP, and T motifs, and we tested their abilities to support telomere maintenance. A total of 18 mutants with substitution in conserved residues (SCR mutants) were generated, each with one or two residues (and in one case, three residues) changed to alanines: IL256, CP261, LE264, F286, IL290, LLP294, MF299, LL316, LL330, DF338, WL341, W367, LL372, IL376, FF380, T384, Y394, and W400. Each mutant is designated by the identity and location of the altered



**FIG. 2. Mutations in the CP, QFP, and T motifs impair telomere maintenance.** Telomere lengths were determined for strains bearing wild type or mutated TERT. The identities of the mutants are indicated at the top of the panel. The location of Y' type telomeres is indicated by brackets. The amplified Y' fragments that are often observed in senescent strains are marked by filled circles.



amino acid residue. Where more than one residue is mutated, the number specifies the position of the first residue of the pair or triplet. For comparison, a mutant with substitutions in non-conserved residues was also made (GK307). 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* (yeast TERT) gene has been disrupted. A similarly tagged wild type *EST2* gene was analyzed in parallel as the control.

All of the strains were first tested for telomere maintenance defects. As predicted and shown in Fig. 2, the majority of SCR mutants exhibited varying degrees of telomere shortening. Based on the degree of shortening, the mutant phenotypes were classified as mild (CP261, LE264, GK307, W367, T384, and W400; less than 50 bp shorter than wild type), intermediate (F286, IL290, MF299, LL316, DF338, WL341, LI372, and Y394; 50–200 bp shorter than wild type), or severe (LLP294, LL330, II376, and FF380; more than 200 bp shorter than wild type). The four strains with severe telomere shortening (LLP294, LL330, II376, and FF380) also exhibited reduced growth on agar plates as evidenced by reduced colony size (data not shown). In addition, these strains display evidence for amplification of the subtelomeric Y' fragment (indicated by filled circles in Fig. 2), which has been shown previously (51) to correlate with senescence and the generation of type I survivors. As predicted, the single non-SCR mutant (GK307) exhibited no defect in telomere maintenance. Surprisingly, five of the SCR mutants (CP261, LE264, W367, T384, and W400) showed either normal telomeres or very mild shortening. However, where it has been tested in other systems, the equivalent residues appear also to be relatively unimportant for telomerase enzyme activity or telomere maintenance (18, 33). We conclude that sequence alignment in the CP, QFP, and T region is useful for identifying functionally important TERT residues.

To determine whether the telomere maintenance defects can be explained by telomerase activity loss or alteration, we precipitated protein A-tagged telomerase from the wild type and mutant strains by adsorption to IgG-Sepharose, and we subjected the Sepharose beads to primer extension analysis. All of the mutant enzymes were tested side-by-side with wild type telomerase using a 16-mer oligonucleotide with non-canonical

telomere repeats (TEL66) as the primer substrate (Fig. 3, A and B). In most cases, comparisons were also made using a 15-nt primer with canonical repeats (TEL15), which yielded very similar results (Fig. 3B, and data not shown). As shown in representative assays and the summary plot (Fig. 3C), there is an excellent correlation between the extent of telomere shortening and telomerase activity reduction. Mutant strains with the most severe telomere length defect contain essentially no telomerase activity (LLP294, LL330, II376, and FF380). For mutants with intermediate telomere defects, the reduction in telomerase activity ranges from ~65% for F286 (which exhibits the least telomere length defect among the intermediate group) to ~95% for DF338, WL341, LI372, and Y394 (which have the most length defect in this group). Mutant strains with mild or no defect had close to or more than 50% of the wild type level of telomerase activity (CP261, LE264, GK307, W367, T384, and W400). None of the mutants with detectable telomerase exhibited an obvious processivity defect (Fig. 3, A and B, and data not shown). Taken together, our results indicate that mutations in the CP, QFP, and T motif impair simultaneously telomere maintenance and telomerase activity but not telomerase processivity.

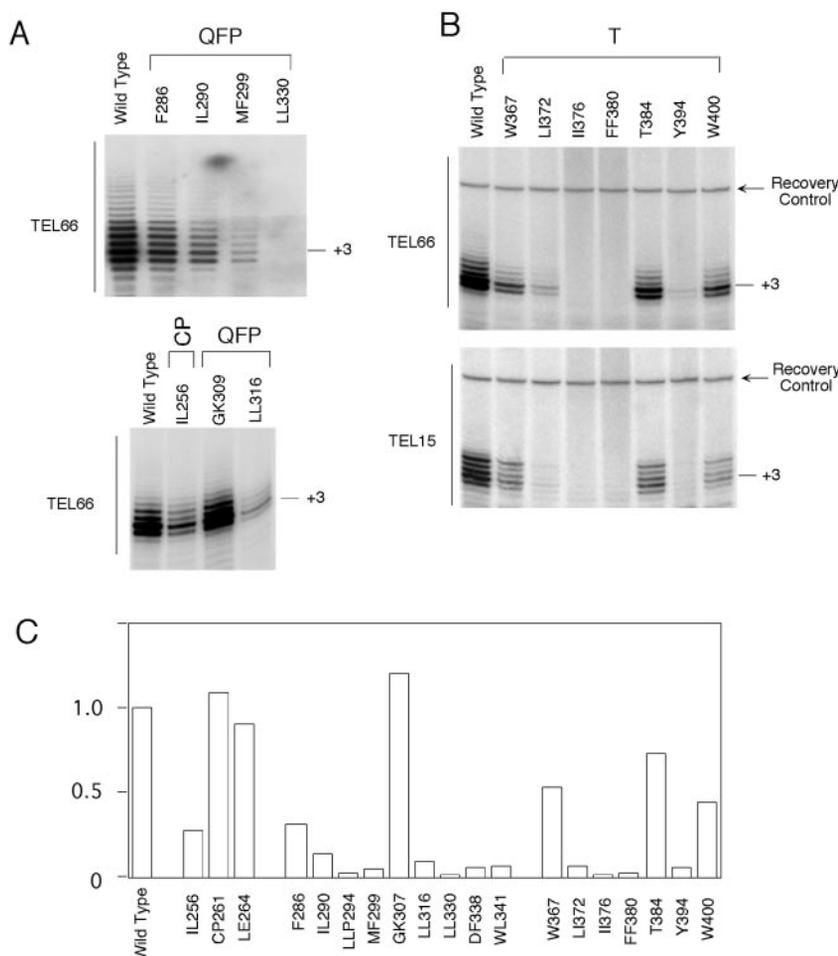
*Mutations in the CP, QFP, and T Motifs Caused a Reduction in the Amount of TERT Protein and TERT-associated TLC1 RNA*—To determine the basis for the activity loss manifested by the SCR mutants, we estimated the levels of TERT protein in wild type and mutant strains using antibodies directed against the protein A tag. The levels of TERT protein in the mutant strains were gauged by comparing the signal obtained from a fixed amount of mutant extract with those from varying amounts of wild type extract. As shown in Fig. 4, A and B, the mutants with significant telomere length defects consistently manifested a reduction in the amount of TERT protein. In addition, the extent of functional loss correlates loosely with the degree of protein reduction. For instance, the mutants with severe telomere shortening all had undetectable levels of TERT protein (LLP294, LL330, II376, and FF380), whereas those with intermediate telomere defects had measurable levels (e.g. IL290, MF299, and LL316).

The amount of TERT-associated TLC1 RNA was measured by IgG-Sepharose adsorption of tagged telomerase followed by

data base codes are as follows: *Tt\_TERT*, *Tetrahymena thermophila* TERT [TERT\_TETTH]; *Ot\_TERT*, *Oxytricha trifallax* TERT [TERT\_OXYTR]; *Ea\_TERT*, *E. aediculatus* TERT [TERT\_EUPAE]; *Mc\_TERT*, *M. crassus* TERT [AAM95622]; *Pc\_TERT*, *P. caudatum* TERT [Q9GRC5]; *Sc\_Est2p*, *S. cerevisiae* TERT [TERT\_YEAST]; *Sp\_TRT1*, *S. pombe* TERT [TERT\_SCHPO]; *Ca\_TERT1*, *Candida albicans* TERT 1 [Q9P8T3]; *Xl\_TERT*, *X. laevis* TERT [Q9DE32]; *Mm\_TERT*, *Mus musculus* TERT [TERT\_MOUSE]; *Ma\_TERT*, *M. auratus* TERT [Q9QXZA]; *Hs\_TERT*, *Homo sapiens* TERT [TERT\_HUMAN]; *Os\_TERT*, *O. sativa* TERT [Q9AU13]; *At\_TERT*, *Arabidopsis thaliana* TERT [Q9SE99]; *Gl\_TERT*, *G. lamblia* TERT [Q9NCP5]; *Pf\_ORF*, *P. falciparum* 3D7 hypothetical protein PF13\_0080 [NP\_705050]; *Py\_TERT*, *P. yoelii* TERT [EAA16235]; *Cp\_TERT*, *C. parvum* TERT [AAK60396]; and *Ec\_TERT*, *E. cuculii* TERT [NP\_596954].

FIG. 3. Mutations in the CP, QFP, and T motifs lead to loss of telomerase activity.

**A**, telomerase from the wild type and various mutant strains was isolated by IgG affinity chromatography and tested in primer extension assays using TEL66 as the primer, and [ $^{32}$ P]dGTP and dTTP as the nucleotides. The identities of the mutations are indicated at the top and the location of the primer +3 (+3) product indicated by horizontal lines. **B**, telomerase from the wild type and various mutant strains was isolated by IgG affinity chromatography and tested in primer extension assays using TEL66 or TEL15 as the primer, and [ $^{32}$ P]dGTP and dTTP as the nucleotides. The identities of the mutations are indicated at the top and the location of the primer +3 (+3) product indicated by horizontal lines. In these assays, a labeled oligonucleotide was precipitated along with the reaction products as a recovery control (arrows). **C**, total DNA synthesis mediated by each mutant enzyme was determined in assays such as those presented in part A and B, normalized against that mediated by the wild type enzyme, and the results plotted. The values are averages of two independent assays.

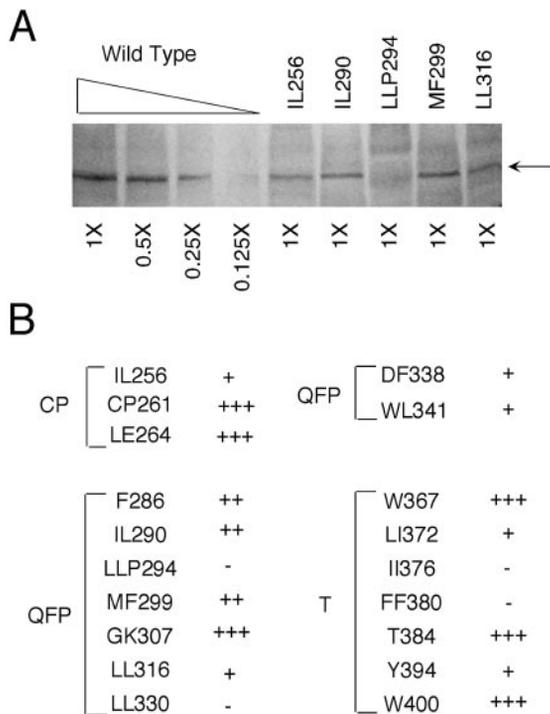


RNase protection analysis (Fig. 5). In most experiments, a single protected species can be detected (Fig. 5A). However, in some experiments, an additional smaller band can be visualized, presumably arising from excessive degradation of the hybrid by RNases (Fig. 5B). Because both bands are due to TLC1 RNA protection, both are included in the calculation of the amount of this RNA. Comparison of the summary plots in Figs. 5C and 3C revealed an excellent correlation between the extent of activity loss and the degree of reduction in the level of TERT-associated TLC1 RNA. Mutants with close to wild type telomerase activity had high levels of TERT-associated RNA (CP261, LE264, GK307, W367, T384, and W400), whereas those with severe defects had little or no associated RNA (LLP294, LL330, II376, and FF380). These results indicate that the loss of telomerase activity in the mutant strains may be accounted for by the loss of TERT and TERT-associated TLC1 RNA. Furthermore, analysis of mutants with appreciable activities suggests that the telomerase complexes containing mutant proteins have specific activities that are comparable with those containing wild type TERT. For example, although the DF338, WL341, LI372, and Y394 mutant each suffered a 90–95% reduction in activity, each also suffered a 90–95% reduction in TERT-associated RNA (Figs. 3C and 5C). The mutant proteins therefore appear to be catalytically competent once associated with RNA.

**Overexpression of the Mutant Proteins Does Not Result in Telomere Shortening**—The concomitant reduction in the level of telomerase activity, TERT protein, and TERT-associated TLC1 RNA suggests that mutations in the CP, QFP, and T motifs affected RNP assembly rather than the *in vivo* and *in vitro* activity of telomerase. To explore this possibility, we over-

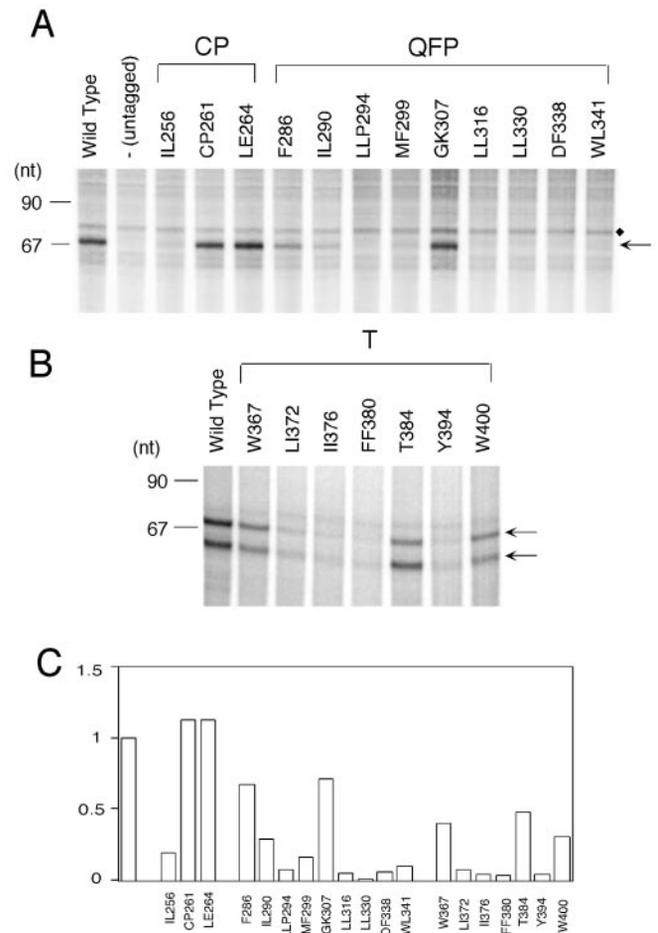
expressed various mutant proteins in a wild type strain background, and we analyzed the lengths of telomeres in the resulting strains. If the mutant proteins are defective in assembly, then they should not compete with endogenous TERT for association with TLC1 RNA and should not act in a dominant-negative fashion even when overexpressed. This was indeed found to be the case. Five SCR mutants of *TERT* (LLP294, MF299, LL330, II376, and FF380) were cloned downstream of the strong and constitutive triose-phosphate isomerase promoter, and the resulting plasmids were introduced into a reference strain containing a native *TERT* gene (W303). Following two re-streaks (~50 generations), chromosomal DNAs were isolated from the clones and analyzed for telomere length alteration (Fig. 6). As a control, a C-terminal truncation mutant (C745), previously shown to be defective in telomerase activity and processivity but not assembly, was tested in parallel (29). As expected, the C745 mutant, when overexpressed, caused dramatic telomere shortening, by ~200 bp. In contrast, none of the SCR mutants (with alterations in the QFP and T motif) was able to act in a dominant-negative fashion with regard to telomere maintenance. We are led to conclude that the major deficiency engendered by the mutations is in telomerase assembly rather than enzyme function.

**The Efficiency of RNP Formation Is Reduced by the Mutations**—In the non-overexpression strains analyzed in Figs. 2–5, the concurrent loss of TERT and TERT-associated RNA can be explained as either a direct or indirect consequence of mutations on protein stability. In the former scenario, the mutations might have caused protein misfolding, leading to accelerated degradation. Fewer TERT molecules would then be available for association with TLC1 RNA. In the latter scenario, the



**FIG. 4. Mutations in the CP, QFP, and T motifs lead to reductions in the level of TERT protein.** A, levels of protein A-tagged TERT in the mutant strains were determined by Western blotting using antibodies directed against protein A and compared with that of the wild type strain. The identities of the mutants are indicated at the *top*, and the relative amounts of extracts loaded indicated at the *bottom*. The amount 1X denotes 400  $\mu$ g of total protein. B, the levels of protein A-tagged TERT in the mutant strains were estimated visually based on assays such as those presented in A and the results summarized. The levels are scored as follows: -, <12.5% of wild type; +, 12.5–25% of wild type; ++, 25–50% of wild type; +++, >50% of wild type. Most of the assays (specifically, the CP and QFP mutants) were performed twice, with very similar results.

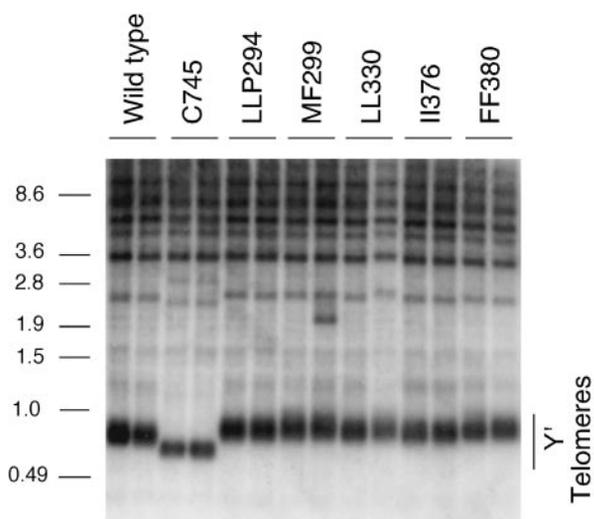
mutations disrupted protein-RNA interaction, resulting in less RNP formation. The unincorporated protein molecules were then preferentially degraded. To distinguish between these possibilities, we examined the efficiency of RNP formation in our overexpression strains. As described earlier, these strains contain, in addition to the native *TERT* gene, a mutated *TERT* (tagged with protein A) that is under the control of the strong triose-phosphate isomerase promoter. For comparison, a yeast strain with a tagged wild type gene driven by the native promoter was tested in parallel. As judged by the Western analysis shown in Fig. 7 (*top panel*), all of the overexpressed mutant proteins accumulated to higher levels than the non-overexpressed wild type protein, which was undetectable in this particular experiment. (The non-overexpressed wild type protein can be detected when the developing time for the blot in the color substrates is increased, as seen in Fig. 4A.) Interestingly, the steady-state level of the LL330 mutant protein is lower than the other mutant proteins, implying some additional defect in protein stability. The levels of TLC1 RNA associated with mutant proteins were measured by IgG-Sepharose adsorption of tagged telomerase followed by RNase protection analysis. Of the mutant proteins, only C745 co-precipitated an increased amount of TLC1 RNA (Fig. 7, *middle panel*, compare *lanes 1* and 7). The other mutants, despite their increased expression, associated with either comparable or reduced levels of RNA in comparison with the non-overexpressed native protein (Fig. 7, *middle panel*, compare *lanes 2–6* with *lane 7*). Furthermore, the efficiency of RNA association correlates with the extent of functional loss *in vivo*. For example, of the five



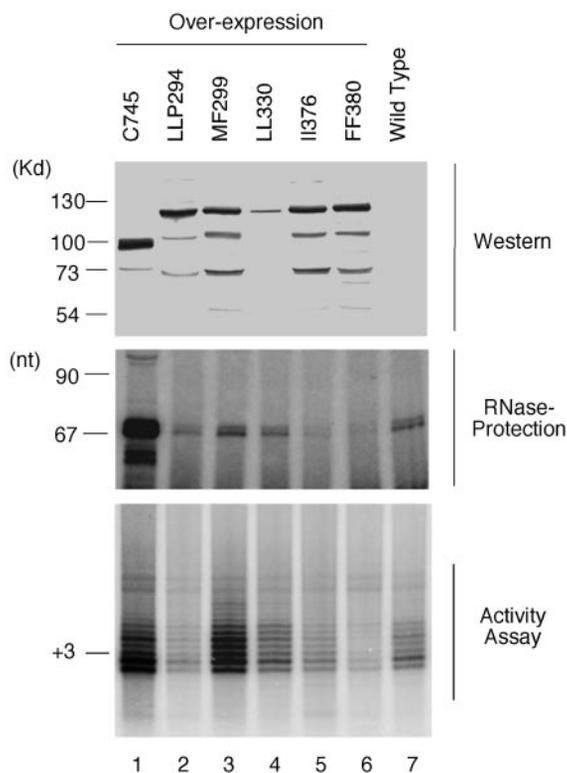
**FIG. 5. Mutations in the CP, QFP, and T motifs cause reductions in the level of TERT-associated TLC1 RNA.** A, the levels of TERT-associated TLC1 RNA in the wild type and mutant strains were determined by RNase protection assays. The identities of the mutants are indicated at the *top*. As a control, an extract was prepared from an untagged strain and subjected to the same analysis. The position of the protected TLC1 fragment is indicated by an arrow on the *right*. A nonspecific band, marked by a diamond, can sometimes be seen in these assays. B, the levels of TERT-associated TLC1 RNA in the wild type and mutant strains were determined by RNase protection assays. The identities of the mutants are indicated at the *top*. The positions of two protected fragments are indicated by arrows on the *right*. C, the levels of TERT-associated TLC1 RNA in the mutant strains relative to the wild type strain were determined by assays such as those shown in A and B, and the average values from two experiments plotted.

overexpressed SCR mutants, MF299 was able to associate with the highest amount of TLC1 RNA when overexpressed. This mutant was also the least impaired in terms of its ability to maintain telomeres in the absence of wild type protein (Fig. 2). These results suggest that the major defect of the mutant proteins is their reduced ability to associate with telomerase RNA.

**Mapping of the 3' End of TLC1 RNA in Telomerase RNP**—In the process of quantifying the amount of TERT-associated TLC1 RNA, we noticed that the length of the probe protected by the RNA appears to be shorter than that predicted by previous analysis. According to a published report (41), the de-adenylated, mature RNA is thought to have 3' ends that cluster near position 1250. If this were true, then the RNase protection assay should yield a protected fragment of ~150 nt. (See Fig. 8A for a schematic illustration of the RNase protection assay.) However, our analysis of telomerase-associated TLC1 RNA in both IgG-Sepharose precipitates and DEAE fractions consistently yielded a shorter fragment of ~70 nt (Figs. 5, 7B, and 8B). To determine whether the short fragment was due to an

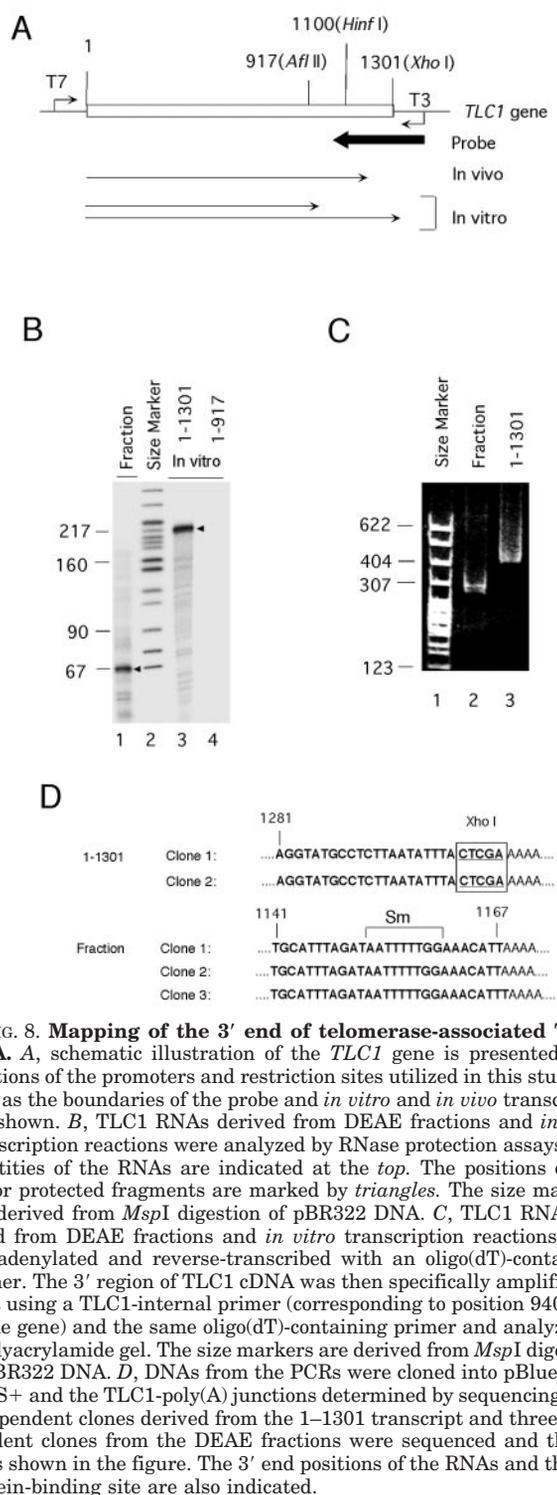


**FIG. 6. Overexpression of proteins with mutations in the QFP and T motifs in a wild type strain background does not cause telomere shortening.** The yeast strain W303 was transformed with a pYX232 plasmid overexpressing a wild type or a mutated TERT. After the transformants were re-streaked twice, chromosomal DNAs were isolated from the strains, digested with *Pst*I, and analyzed for telomere lengths. The identities of the overexpressed proteins in the transformants are indicated at the top.



**FIG. 7. Analysis of the efficiency of RNP assembly in the over-expression strains.** W303-derived strains that overexpress various TERT mutants were analyzed for TERT protein levels (top panel), TERT-associated TLC1 RNA (middle panel), and TERT-associated telomerase activity (bottom panel) by Western, RNase protection, and primer extension assays, respectively. For comparison, a strain carrying a tagged wild type *TERT* gene under the control of the native promoter was tested in parallel (lane 7). The identities of the overexpressed TERT mutant proteins are indicated at the top.

unstable hybrid that can be cleaved internally by the RNases, we subjected an *in vitro* transcript that encompasses residues 1–1301 of the TLC1 RNA to the same assay. As shown in Fig. 8B, with this RNA, only a 200-nt fragment can be detected.



**FIG. 8. Mapping of the 3' end of telomerase-associated TLC1 RNA.** A, schematic illustration of the *TLC1* gene is presented. The locations of the promoters and restriction sites utilized in this study, as well as the boundaries of the probe and *in vitro* and *in vivo* transcripts, are shown. B, TLC1 RNAs derived from DEAE fractions and *in vitro* transcription reactions were analyzed by RNase protection assays. The identities of the RNAs are indicated at the top. The positions of the major protected fragments are marked by triangles. The size markers are derived from *Msp*I digestion of pBR322 DNA. C, TLC1 RNAs derived from DEAE fractions and *in vitro* transcription reactions were polyadenylated and reverse-transcribed with an oligo(dT)-containing primer. The 3' region of TLC1 cDNA was then specifically amplified by PCR using a TLC1-internal primer (corresponding to position 940–965 of the gene) and the same oligo(dT)-containing primer and analyzed in a polyacrylamide gel. The size markers are derived from *Msp*I digestion of pBR322 DNA. D, DNAs from the PCRs were cloned into pBluescript II KS+ and the TLC1-poly(A) junctions determined by sequencing. Two independent clones derived from the 1–1301 transcript and three independent clones from the DEAE fractions were sequenced and the results shown in the figure. The 3' end positions of the RNAs and the Sm protein-binding site are also indicated.

Moreover, the use of a 1–917 transcript, missing the region of TLC1 complementary to the probe, completely abolished the signal. Taken together, our results indicate that the RNase protection assay correctly maps the 3' end of the *in vivo* and *in vitro* RNA.

To further refine the mapping analysis, we sequenced the 3' end of the *in vivo* RNA using a protocol developed previously (41). The RNAs in the fraction were extended by poly(A) polymerase and reverse-transcribed into cDNA using an oligo(dT)-containing primer. The 3' region of TLC1 cDNA was then specifically amplified by PCR, cloned into a plasmid vector, and sequenced. As a control for the reliability of the pro-

cedure, we analyzed the 3' end of the 1–1301 transcript in parallel. As shown in Fig. 8C, The PCR fragments for the *in vivo* and *in vitro* RNA differ in size by ~130 bp, again consistent with the RNase protection study. Sequence analysis of independent clones indicate that the *in vivo* RNA terminates at either position 1167 or 1168, whereas the *in vitro* RNA terminates in a partial *Xho*I site after position 1301, precisely as predicted (Fig. 8D). We conclude that the major 3' end for telomerase-associated TLC1 RNA is considerably more upstream of the major polyadenylation sites (~1250), suggesting that during telomerase maturation, the RNA undergoes both de-adenylation and nucleolytic cleavage.

#### DISCUSSION

*The Functions of the CP, QFP, and T Motifs*—We have shown in this study that three of the four conserved N-terminal motifs of yeast TERT are required for efficient telomerase ribonucleoprotein assembly but not for enzyme function. Evidence in favor of this proposition includes the following: 1) the simultaneous loss of telomerase activity, TERT protein, and TERT-associated RNA in the mutant strains; 2) the inability of the mutant proteins to act in a dominant-negative fashion when overexpressed in the presence of wild type protein; 3) the failure of the overexpressed mutant proteins to associate with telomerase RNA efficiently; and 4) the close quantitative correlation between telomerase activity and telomerase-associated RNA in the mutant and overexpression strains. Friedman and Cech (52) have shown earlier that two 10-amino acid substitutions in region III of yeast TERT (corresponding to the QFP motif) impair association with TLC1 RNA. Our results are in agreement and highlight the importance of conserved TERT residues in mediating telomerase assembly.

The mechanistic basis for the observed assembly defect is not clear from the present analysis; telomerase biogenesis entails multiple steps, several of which can potentially be disrupted by the mutations. Based on earlier analysis from several systems, two plausible hypotheses may be considered. First, direct physical interaction between yeast TERT and TLC1 RNA may be impaired by the mutations. Consistent with this idea, the CP, QFP, and T motifs of *Tetrahymena* and human TERT have been demonstrated previously (33, 53–55) to be required for binding telomerase RNA *in vitro*. Second, relevant intracellular localization of yeast TERT may be impaired by the mutations, leading to reduced assembly. Consistent with this latter possibility, residues in the CP and T motifs of human TERT are believed to mediate nucleolar localization, and nucleolar localization has been proposed to be an important step in telomerase biogenesis (55–57). Further studies will be necessary to distinguish between these (non-mutually exclusive) hypotheses.

The phenotypic consequences of mutations in the CP, QFP, and T motifs contrast sharply with those of the GQ motif. For instance, some of the mutations in the GQ region adversely affect telomere maintenance without affecting telomerase activity (32, 52, 58). In addition, alteration of conserved residues in the GQ motif can result in mutants that act in a dominant-negative fashion when overexpressed in the presence of wild type protein, indicative of some defect in enzyme function rather than assembly (32). Finally, in complementation experiments *in vitro*, telomerase activity can be reconstituted by mixing an active-site mutant of human TERT with one that bears mutations in the GQ motif (but not in the other three motifs) (59). Taken together, these results strongly imply a separate function(s) for the most N-terminal GQ motif, which apparently resides in a distinct physical domain (32).

*Evolutionary Conservation of the TERT N-terminal Motifs*—The evolutionary conservation of TERT N-terminal motifs is

remarkable and is consistent with crucial function(s) for these motifs in telomerase assembly and/or catalysis. As described earlier, an interesting feature of these motifs is the preponderance of hydrophobic/aromatic residues at conserved positions. If the CP, QFP, and T motifs are indeed involved in direct RNA-protein binding, as suggested earlier, then it is tempting to speculate that this binding may occur primarily through base-stacking interactions. It is also tempting to speculate that common features of the telomerase RNAs may be recognized by these motifs. Conversely, the apparent lack of these motifs in *C. elegans* TERT suggests that the nature of RNA-protein interaction may be quite distinct for this telomerase.

*The Processing of Telomerase RNA*—The synthesis and maturation of telomerase RNA appears to be a poorly conserved process. For example, in ciliates, telomerase RNA is evidently transcribed by RNA polymerase III, whereas in yeast and mammals, the same function is mediated by RNA polymerase II (41, 60–62). Human telomerase RNA appears to be processed by the same factors that process small nucleolar RNAs with the box H/ACA motif (62, 63). In contrast, the *Saccharomyces* TLC1 RNA is bound by Sm proteins and requires the same proteins for accumulation (64). Understanding the precise pathways of RNA maturation is clearly an important aspect of understanding telomerase biogenesis. An earlier study of TLC1 RNA processing indicates that this RNA is transcribed by RNA polymerase II and initially polyadenylated (41). The majority of RNA then becomes de-adenylated, and this appears to be the predominant form that is present in telomerase RNP. With the exception of the poly(A) tail, the two RNA populations are believed to have the same 3' ends, clustering around position 1250. However, our current study suggests that the majority of telomerase-associated TLC1 RNA has a more upstream 3' end, around position 1167. TLC1 RNA may therefore be subjected to additional cleavage events during maturation and RNP assembly. Curiously, the newly discovered 3' end is only a few nucleotides downstream of the Sm protein-binding site (Fig. 8D), suggesting that the cleavage activity may be regulated by the Sm protein complex (64). Such a cleavage step appears to be common to small nuclear RNAs that associate with Sm proteins, implying a further parallel between the processing of these RNAs and TLC1 (65).

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