Telomerase can act as a template- and RNA-independent terminal transferase

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Telomerase is a special reverse transcriptase that extends one strand of the telomere repeat by using a template embedded in an RNA subunit. Like other polymerases, telomerase is believed to use a pair of divalent metal ions (coordinated by a triad of aspartic acid residues) for catalyzing nucleotide addition. Here we show that, in the presence of manganese, both yeast and human telomerase can switch to a template- and RNA-independent mode of DNA synthesis, acting in effect as a terminal transferase. Even as a terminal transferase, yeast telomerase retains a species-dependent preference for GT-rich, telomere-like DNA on the 5′ end of the substrate. The terminal transferase activity of telomerase may account for some of the hitherto unexplained effects of telomerase overexpression on cell physiology.

reverse transcriptase | telomere | divalent metal ion

Telomerase is a ribonucleoprotein responsible for the synthesis of one strand of the telomere terminal repeat. The catalytic core of telomerase consists minimally of two components: an RNA in which the template is embedded (named TLC1 in the budding yeast *Saccharomyces cerevisiae*), and a reverse transcriptase (RT)-like protein that mediates catalysis (named TERT in general and Est2p in yeast) (1–4). Telomerase RNAs from different organisms are divergent in sequence but share conserved secondary structural elements (5, 6). Many mutations in nontemplate regions of telomerase RNA reduced enzyme activity, suggesting that these regions contribute important catalytic functions [although the precise nature of the contribution(s) is not clear] (7–9). Other RNA structures act to define the boundary of the template segment that supports reverse transcription (10, 11). Thus, the RNA subunit serves multiple essential roles in the course of a normal telomerase reaction. The TERT protein is well conserved in evolution and comprises a central RT domain that is flanked on both the N- and C-terminal side by telomerase-specific motifs (4, 12, 13). Mutational analysis indicates that these motifs are likely to mediate binding to telomeric DNA and telomerase RNA. Direct recognition of telomeric DNA by TERT is believed to be sequence-dependent and to allow telomerase to catalyze the addition of multiple repeats without dissociation from the DNA (13, 14).

In unicellular organisms, telomerase is constitutively active and required for the long-term proliferation of cells. In some multicellular organisms, including humans, telomerase is repressed in normal somatic tissues but activated in cancer cells, and is believed to promote tumor growth (15). Although the two core components of telomerase are generally assumed to act in concert to replenish telomeres, there is increasing evidence that the TERT protein can have physiologic effects that are independent of telomere length and telomerase RNA (16–19). For instance, in mice, TERT overexpression in the absence of telomerase RNA was reported to have an inhibitory effect on tumorigenesis and wound healing (20). The mechanisms for such effects are currently not understood.

All polymerases are believed to be metal-dependent, and various metal ions have been shown to influence the activity and fidelity of nucleic acid synthesis (21). However, their effects on telomerase reverse transcriptase have not been reported. Because the integrity of telomere sequence is important for the assembly of telomeric nucleoprotein complex and chromosome stability (22–24), the potentially mutagenic effects of metal ions on telomerase-mediated DNA synthesis may have important physiologic implications. In this report, we show that the TERT protein has a hitherto unsuspected, evolutionarily conserved terminal transferase (TT) activity that can be revealed in the presence of manganese. This activity depends on the active site of the RT domain but not any region of telomerase RNA. The biochemical and physiologic ramifications of our findings are discussed.

Materials and Methods

Yeast Strains and Plasmids. The *pmr1*-Δ strain and the isogenic wild-type strain (BY4741) were purchased from Open Biosystems (Huntsville, AL), and the identity of the mutant was confirmed by PCR. The *tlc1*-Δ strain was constructed in the W303a background by using a PCR-generated disruption cassette that precisely replaces the *TLC1* gene with a *LEU2* marker. The strain was subsequently transformed with pSE-Est2-C874, which carries a protein A-tagged Est2p and allowed Est2p to be purified on IgG-Sepharose.

Analysis of Yeast Telomeres and Telomerase. The protocols for yeast telomere length analysis, extract preparation, and telomerase activity assays have all been described (25, 26).

Analysis of Human Telomerase. The method for human TERT (hTERT) expression in reticulocyte lysate (RRL) has been described (27). RRL samples were treated with RNase for 30 min before being tested in direct primer extension assays using 2.5 μM biotinylated $(G_2T_2AG)_3$ primer. Earlier analysis indicates that this RNase treatment is sufficient to abolish all telomerase activity in a conventional assay that contains only magnesium.

Results

We first examined the effect of different divalent cations on partially purified yeast telomerase (*Saccharomyces cerevisiae*) in primer extension assays, and found that quite a few metals including Ca^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} can significantly repress telomerase activity *in vitro* at mM concentrations in the presence of 2.5 mM Mg^{2+} (Fig. 5, which is published as supporting information on the PNAS web site). Interestingly, the effect of at least one metal ion appears to be species-specific: Ca^{2+} can severely inhibit telomerase from *S. cerevisiae*, but had at most a minor effect on the closely related enzyme from

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Abbreviations: RT, reverse transcriptase; TT, terminal transferase.

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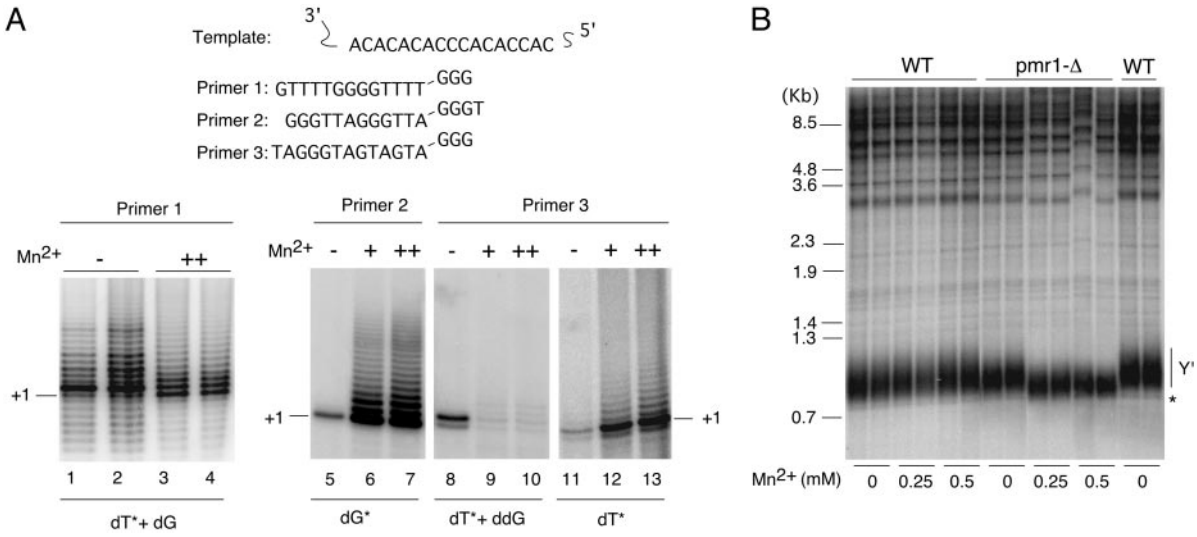

Fig. 1. Manganese can influence the activity and fidelity of *S. cerevisiae* telomerase *in vitro* and affect telomere maintenance *in vivo*. (*A*) *S. cerevisiae* telomerase was isolated on IgG-Sepharose as described (26), and subjected to primer extension assays in the presence of 0 mM (−), 1 mM (+), or 3 mM (++) Mn^{2+} as indicated above the panel. All reactions also contained 2.5 mM Mg^{2+}, of which 0.5 mM were contributed by the bead suspension and 2 mM were contributed by the assay buffer. The primers, labeled nucleotides (indicated by asterisks), and unlabeled nucleotides included in the reactions are listed above and below the panel. The sequence of yeast telomerase RNA template, the primer sequences, and the presumed alignments between the template and primers are illustrated at the top. (*B*) Two isolates each of a *pmr1*-Δ strain and the isogenic wild-type BY4741 strain (Open Biosystems) were grown in liquid SD medium supplemented with varying concentrations of $MnCl_2$ (as indicated at the bottom) for 30 generations. Note that the SD medium itself contains 2 mM Mg^{2+} and 2.5 μM Mn^{2+}. Chromosomal DNAs were isolated from the strains by Smash and Grab, and the telomere restriction fragments were analyzed by Southern blots (26). The location of Y′ telomeres is indicated by a vertical bar at the right of the panel. A nontelomeric fragment (asterisk) hybridized weakly to the probe and serves conveniently as a migration standard for DNA samples, which show slightly reduced mobility near the right side.

Candida albicans (Fig. 5). Thus, not all metals are likely to interact with conserved telomerase residues.

Manganese Altered the Fidelity of Yeast Telomerase. We next assessed the impact of adding Mn^{2+}, whose mutagenic effects on other polymerases are most extensively characterized (21, 28). Interestingly, in the presence of the standard combination of 0.2 μM dTTP and 50 μM dGTP, which enables yeast telomerase to add ≈15 nucleotides (29) to the telomeric primer, the inclusion of 3 mM Mn^{2+} resulted in a slight reduction in total DNA synthesis and a mild reduction in the processivity of telomerase (Fig. 1*A*, lanes 1–4). Remarkably, when either dGTP or dTTP alone was included in the *in vitro* reaction, telomerase extended the primer by a single nucleotide in the absence of Mn^{2+} (as would be expected based on the primer/template alignment and the template sequence) (Fig. 1*A*, lanes 5 and 11), but by up to almost 20 nucleotides in the presence of Mn^{2+} (lanes 6, 7, 12, and 13). Moreover, with the combination of labeled dTTP and unlabeled ddGTP, telomerase faithfully executed the addition of two nucleotides in the absence of Mn^{2+}, but became much less active in the presence of Mn^{2+} (lanes 8–10). All of these observations are consistent with a significant loss of fidelity by telomerase in the presence of Mn^{2+}. In particular, telomerase appears to be more prone to slippage synthesis or less reliant on template-nucleotide base-pairing during polymerization. The inhibitory effect of ddGTP in the presence of Mn^{2+}, for example, may be attributed to competition of unlabeled ddGTP with labeled TTP at the nucleotide binding site that would, under high-fidelity conditions, only accommodate the latter nucleotide.

Telomerase Can Act as a TT. To address the possibility that the requirement for proper base-pairing between template and nucleotide may be substantially relaxed in the presence of Mn^{2+}, we pretreated telomerase with RNase A and subjected the resulting preparation to primer extension analysis. As expected, in the presence of Mg^{2+} alone, the RNase pretreatment completely abolished the extension reaction (Fig. 2*A*, lanes 1 and 2). In contrast, in the presence of Mn^{2+} alone or Mn^{2+} and Mg^{2+}, the signals in the dGTP only reactions were reduced by only 2-fold after RNase pretreatment (lanes 3–6). These results indicate that telomerase is at least partially template-independent in the presence of Mn^{2+}, and prompted us to examine the enzyme's ability to incorporate labeled dATP and dCTP, two nucleotides that are not encoded by the RNA template. As shown in Fig. 2*B*, the ability of telomerase to add dCTP and dATP to the starting primer was indeed greatly enhanced by the addition of Mn^{2+} (lanes 1–3 and 5–7). Moreover, the activity was even higher with enzyme that had been pretreated with RNase A (lanes 4 and 8). Thus, even though the RNA template was dispensable for nucleotide addition in the presence of Mn^{2+}, it appears still to act as a fidelity factor, mitigating against the incorporation of noncomplementary nucleotides. Titration studies indicate that the dCTP-incorporating activity of telomerase was minimal below 0.3 mM Mn^{2+} but became greatly enhanced at higher concentrations (data not shown). Pretreatment of the telomerase preparation with DNase I had no effect on its RNase-independent, Mn^{2+}-dependent activity, suggesting that telomerase did not use a contaminating DNA as the template (Fig. 6, which is published as supporting information on the PNAS web site). In contrast to Mn^{2+}, none of the other metal ions mentioned earlier were able to support the incorporation of dCTP (data not shown). Taken together, these results support the notion that, in the presence of Mn^{2+}, yeast telomerase can switch to a template-independent mode of DNA synthesis, acting as a TT.

The TT Activity Is Mediated by TERT. The apparent TT activity of yeast telomerase cannot be attributed to a contaminating enzyme. First, aphidicolin, an inhibitor of the major DNA polymerases in yeast, had no effect on the terminal transferase activity (Fig. 3*A*). Second, the TT activity was completely absent from a telomerase preparation in which two of the three catalytic

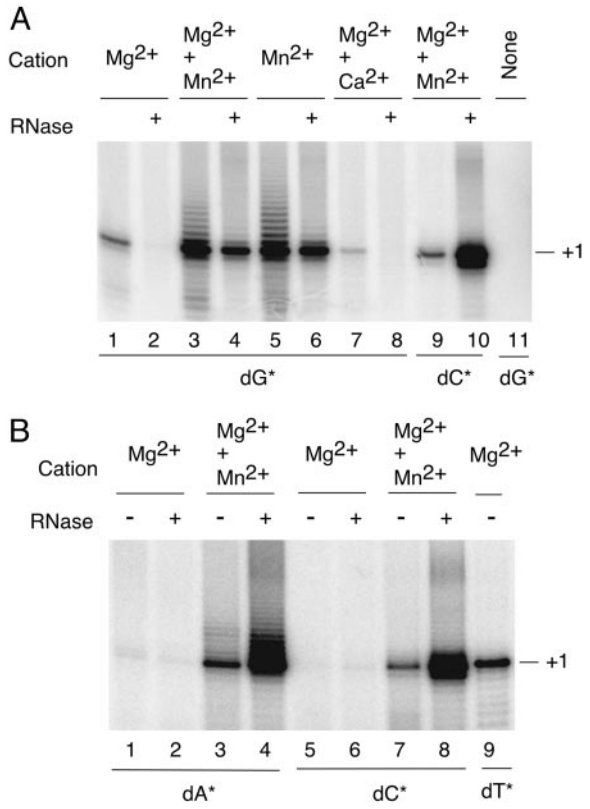

Fig. 2. Yeast telomerase becomes RNase-insensitive and template-independent in the presence of Mn^{2+}. *S. cerevisiae* telomerase was isolated on IgG-Sepharose, incubated with or without 0.3 μg/μl RNase A, and subjected to primer extension assays (using primer 1 in Fig. 1) in the presence of varying combinations of metal ions as indicated above the panels. The concentrations of the ions are as follows: 3 mM Ca^{2+}; 2 mM Mg^{2+}, 3 mM Mn^{2+}. The labeled nucleotides included in the reactions are listed below the panels. Note that, in standard telomerase preparations, the IgG-Sepharose beads were resuspended in a buffer containing 1.2 mM Mg^{2+}. However, in this particular set of assays, the Sepharose beads were washed in buffers without Mg^{2+}. Thus, all of the divalent cations in the reactions were added exogenously. Also note that a +1 product can be observed in lane 1 of *A* (and in an identical reaction in Fig. 3*B*). This would not be predicted if accurate alignment between the template and primer has taken place. Most likely, the product was due to a slight misalignment such that the 3′-most two Gs of the primer base paired with the 3′ two Cs of the triple Cs in the template. Because the product was completely RNase-sensitive, it was not consistent with a ''terminal transferase'' mode of synthesis. Telomeres containing four consecutive Gs can be detected at low frequencies in yeast, suggesting that this misalignment can occur to some extent *in vivo* (48).

aspartic acids of Est2p (30) have been mutated to alanine (Fig. 3*B*). Another Est2p mutant with a substitution near the RT nucleotide-binding pocket (named R450K; ref. 31) likewise had greatly diminished TT activity (data not shown), suggesting that the TT activity is mediated by the active site of RT. We also addressed the possibility that the telomerase RNA component in our preparation may not have been completely removed by RNase treatment, and that some residual RNA moiety may be important for the TT activity. Telomerase was isolated from both a wild-type and a *tlc1*-Δ strain (in which the template RNA gene is deleted), and assayed in parallel. As shown in Fig. 3*C*, in the TT assays, the two enzyme preparations exhibited comparable levels of activity, implying that the entire telomerase RNA is dispensable for the activity. The TT activity of telomerase is apparently conserved in evolution. For instance, the telomerase from *Candida albicans* also becomes template-independent in the presence of Mn^{2+} (Fig. 7, which is published as supporting information on the PNAS web site). Furthermore, the human telomerase catalytic protein hTERT that was expressed in the absence of telomerase RNA in reticulocyte lysates can also mediate the addition of a nontelomeric nucleotide (dC) in a Mn^{2+}-dependent fashion (Fig. 3*D*). Like the yeast TT activity, the human activity requires at least one of the catalytic Asp residues (D868).

The TT Activity Exhibits Preference for Telomere-Like Substrates. Telomerase has been shown to exhibit a preference for elongating GT-rich telomere-like oligonucleotides. Both the RNA template and some putative DNA-binding domain(s) of the telomerase protein are thought to contribute to this preference (32–35). As shown in Fig. 4, even in the TT mode of DNA synthesis, telomerase manifested a strong preference for extending GT-rich oligonucleotides (Fig. 4 *A* and *B*, lanes 4–12). This result further rules out the possibility that a non-sequence-specific contaminating nucleotidyl transferase is responsible for the observed activity. It also indicates that the DNA-binding domain(s) of yeast telomerase has an intrinsic, RNA-independent preference for GT-rich sequences. The protein-mediated sequence recognition appears to be directed toward the 5′ end of the DNA substrate: primers containing GT-rich sequences on the 5′ end consistently supported higher levels of DNA synthesis in the TT assay than comparable primers with GT-rich sequences on the 3′ end (Fig. 4 *A*, *C*, and *D*). As expected, longer GT-rich tracts supported higher levels of activity. These findings provide unequivocal support for the longstanding notion that sequence-dependent recognition of DNA by telomerase is at least bipartite, with the telomerase RNA and protein mediating binding to the 3′ and 5′ end of the DNA, respectively (14). In contrast to *Saccharomyces*, the *Candida* telomerase, although capable of terminal transferase activity, did not exhibit a preference for GT-rich primers, consistent with the atypical composition (non GT-rich) of the *Candida* telomere repeat (Fig. 8, which is published as supporting information on the PNAS web site). Thus, the sequence specificity of the telomerase protein is likely to have evolved in relation to the cognate telomere repeat.

Elevated Intracellular Manganese Induced Telomere Shortening. To determine whether Mn^{2+} can influence telomerase function *in vivo*, we analyzed the telomeres of a yeast mutant (named *pmr1*-Δ) with a defect in Mn^{2+} transport, and hence elevated intracellular Mn^{2+} (36). When the strain was placed in 0.25 or 0.5 mM Mn^{2+}, its growth was impaired (data not shown) and its telomeres were found to be shorter by ≈50–100 bp than those of the wild-type strain (Fig. 1*B*), suggesting that Mn^{2+} can affect telomerase function *in vivo*. Telomere shortening was observed in a second *pmr1*-Δ clone with a different strain background (data not shown). However, the extent of telomere shortening is relatively small and unlikely to be responsible for the growth impairment (26). In addition, because of the dependence of many cellular enzymes on metal ions, we evidently cannot rule out the possibility that the shortening was due to some other effects of Mn^{2+}. The intracellular free Mn^{2+} concentration in yeast has been estimated to be in the low μM range, even for the *pmr1*-Δ strain (36). Thus, it seems unlikely, given the aforementioned titration study, that yeast telomerase will be able to act as a robust terminal transferase *in vivo*, even in the mutant. Nevertheless, a low level of activity may be sufficient to induce physiologic consequences.

Discussion

Aspects of our findings are far from unprecedented. For example, ciliate telomerases have been reported to perform iterative dG addition in the presence of this single nucleotide (although in these studies the RNA requirements were not reported) (37, 38). Furthermore, a variety of DNA and RNA polymerases, including reverse transcriptases, are known to mediate template-

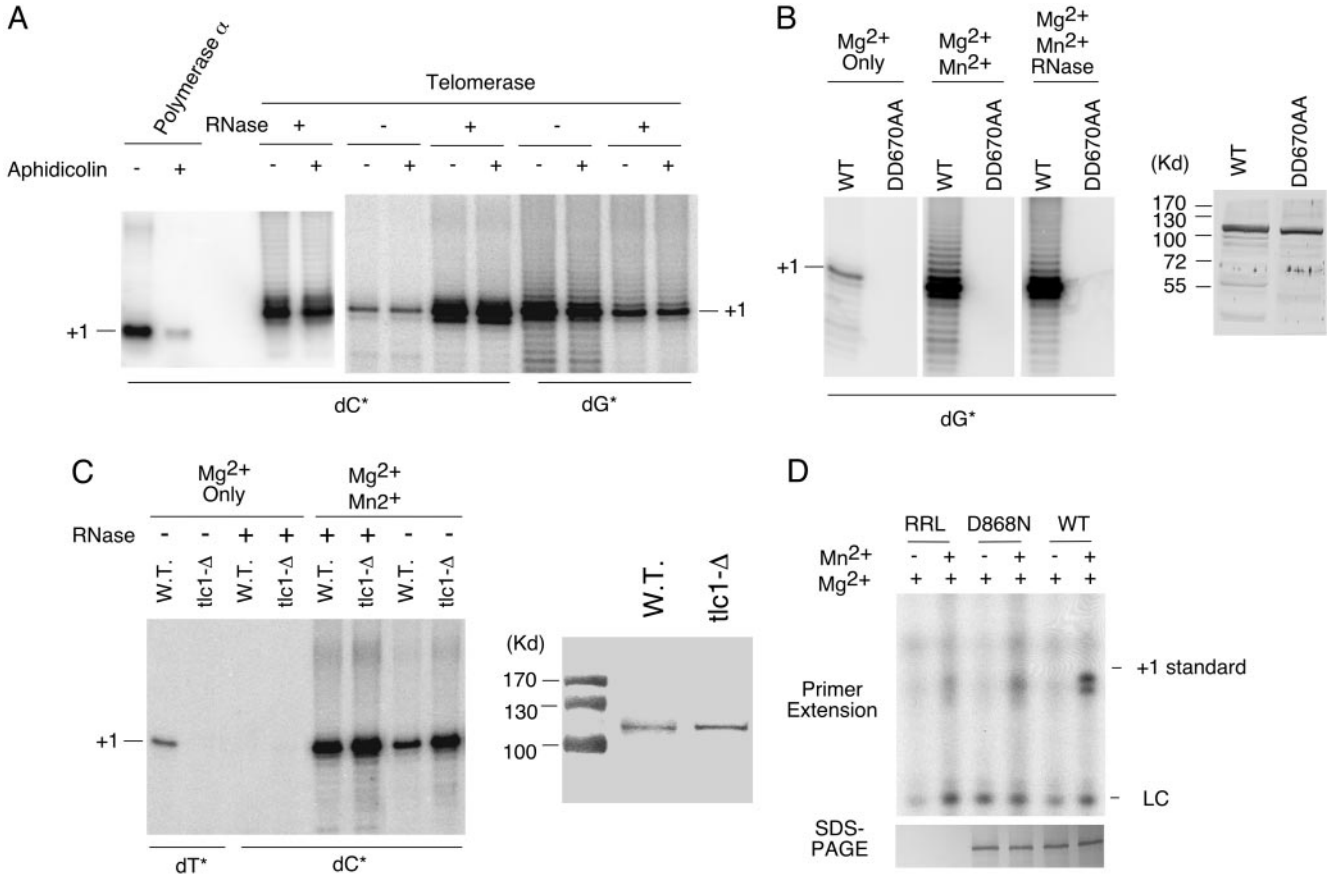

Fig. 3. The functional requirements for the TT activity and its evolutionary conservation. (*A*) (*Right*) *S. cerevisiae* telomerase was isolated on IgG-Sepharose, incubated with or without 0.3 μg/μl RNase A, and subjected to primer extension assays using primer 1 (Fig. 1) in the absence or presence of 10 μg/ml aphidicolin as indicated above the panel. The labeled nucleotides included in the reactions are listed below the panel. (*Left*) The effect of 10 μg/ml aphidicolin on DNA polymerase α (from *Schizosaccharomyces pombe*; a gift of J. Hurwitz) and RNase-treated telomerase was tested and displayed on the same gel. The substrate for the pol α assay was a template-primer duplex (5′-ACTTCTTGGTGTACGGATGTCTA-3′ and 5′-TAGACATCCGTACA-3′) that supports the addition of a single dC nucleotide to a 12-nt primer in the presence of dCTP alone. (*B*) Yeast telomerase with either a wild-type Est2p (WT) or RT active site mutations (DD670AA) was isolated on IgG-Sepharose. The resulting preparations were subjected to Western (*Right*) or primer extension (*Left*) analysis. To detect terminal transferase activity, telomerase was incubated with or without RNase and tested in the presence of 3 mM Mn^{2+} as indicated at the top. The primer used in these assays was primer 1 as listed in Fig. 1. (*C*) Yeast telomerase was isolated from a wild-type or *tlc1*-Δ strain and tested for template-dependent and -independent extension activities (*Left*). The labeled nucleotides included in the reactions are indicated below the panel. The quantities of the Est2p protein in the two preparation were also analyzed by Western blot (*Right*). The primer used in these assays was primer 1 as listed in Fig. 1. (*D*) The wild-type hTERT and an active site mutant (WT and D868N) were expressed in rabbit reticulocyte lysates in the absence of hTR, and tested for their ability to label a human telomere repeat primer $(G_2T_2AG)_3$ in the presence of labeled dCTP only. The reactions were performed in the presence of 2 mM Mg^{2+} alone or 2 mM Mg^{2+} and 3 mM Mn^{2+}, and the products were analyzed in a denaturing gel along with a labeled oligonucleotide that serves as the loading control (LC). This set of assays were repeated two more times, and the Mn^{2+}-dependent doublet was reproducibly observed in the WT but absent in the reticulocyte lysate-alone and D868N reactions. The smaller than +1 product is presumably due to the nucleolytic cleavage activity of human telomerase (49, 50). Such products are also evident in some yeast telomerase reactions (e.g., lane 10 in Fig. 2*A*). Wild-type and mutant hTERTs were visualized by SDS/PAGE and autoradiography, as shown at the bottom.

independent nucleotide addition under some conditions (39–41). Nevertheless, as discussed below, our discovery of telomerase-mediated terminal transferase activity has both biochemical and physiologic implications.

From the biochemical perspective, our finding addresses a longstanding question in telomerase enzymology, i.e., the role of the RNA in catalysis. It has been argued, based on a substantial body of evidence, that nontemplate regions of the telomerase RNA mediate important catalytic functions (7, 42–45). Our findings indicate that the entire RNA subunit is dispensable for the covalent chemistry of telomerase (although it is entirely plausible that the RNA can modulate protein conformation and/or directly contribute to substrate recognition). Furthermore, our TT assay offers a simple and sensitive method of analyzing the protein-dependent recognition of telomeric DNA free of the confounding effect of telomerase RNA.

From the physiologic perspective, our results suggest a potential mechanism for certain unexplained experimental findings and a potential antineoplastic strategy. There is increasing evidence, for example, that overexpression of mammalian TERT can have physiologic consequences (e.g., inducing senescence and modulating tumorigenesis) that cannot be rationalized by its effect on telomere lengths (16–19). We suggest that, because telomerase RNA is likely to be an important fidelity factor, overexpression of the telomerase protein alone may unmask its normally cryptic TT activity, resulting in the synthesis of nontelomeric sequences and potentially detrimental effects on cell physiology. However, it is not known whether the intracellular concentration of Mn^{2+} in mammalian cells can reach a level to support the TT activity of telomerase in normal or pathologic conditions. On the other hand, our results do indicate an intrinsic ability of hTERT to

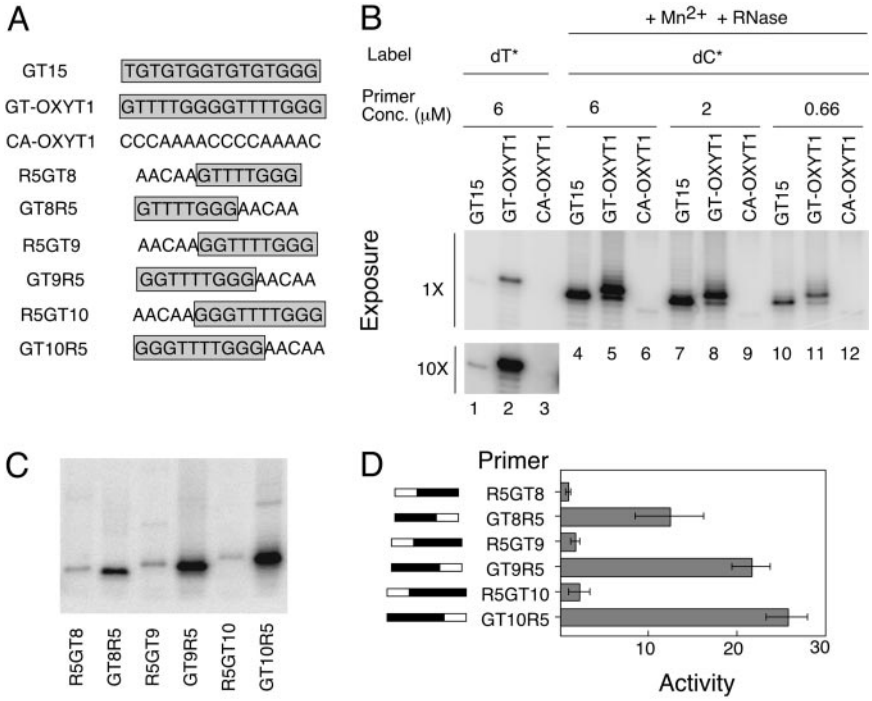

Fig. 4. The sequence preference for yeast telomerase in TT assays. (*A*) The sequences of the primers used to analyze the recognition property of yeast telomerase in TT assays are shown. The TG-tracts in the primers are boxed and shaded. (*B*) Yeast telomerase was isolated on IgG-Sepharose and tested for template-dependent and -independent primer extension activities using three different primers at varying concentrations as indicated above the panel. The labeled nucleotides included in the reactions are indicated below the panel. Note that in the RT mode of synthesis (lanes 1–3), the TG15 primer supported less DNA synthesis than the TG-OXYT1 primer (25). This was most likely due to a stronger interaction between telomerase and TG15 (as a consequence of a long RNA–DNA hybrid), which reduced the extent of enzyme turnover. This difference is absent in the TT mode of synthesis. (*C*) Yeast telomerase was isolated on IgG-Sepharose, treated with 0.3 mg/ml RNase A, and tested for primer extension in the presence of Mn^{2+} and labeled dCTP. The primer sequences are shown in *A*. (*D*) The assays shown in *C* were repeated twice, and the results were quantified. Average activities and standard deviations were calculated and plotted. The primers are also shown schematically with the filled bars designating the locations of the TG-tracts.

act as a terminal transferase. Thus, it may be possible to identify small molecules that trigger this activity even in the absence of high Mn^{2+} concentrations. The introduction of abnormal sequences at chromosomal ends through the expression of mutant telomerase RNAs has been shown to induce cell death, and is proposed to be a valid strategy for cancer therapy (24). Our finding suggests an alternative way of introducing abnormal sequences, i.e., by switching on the terminal transferase activity of telomerase. Clearly, further studies will be necessary to determine the physiologic significance and medical potential of this normally cryptic activity of telomerase.

As a final remark, we note that telomerase was initially named "telomere terminal transferase" (46, 47). Our results argue that this original name is prescient in anticipating an inherent attribute of a versatile enzyme.

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