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 template-independent transferase activity of telomerase may account for some of the hitherto unexplained effects of telomerase overexpression on cell physiology.

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-Est2p-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).

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 Ca2+, Cd2+, Zn2+, Cu2+, Ni2+, and Co2+, can significantly repress telomerase activity in vitro at mM concentrations in the presence of 2.5 mM Mg2+ (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: Ca2+ can severely inhibit telomerase from S. cerevisiae, but had at most a minor effect on the closely related enzyme from S. pombe. This discrepancy suggests that telomerase RNA from different species can have important different regulatory properties.

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

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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\textsuperscript{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\textsuperscript{2+} resulted in a slight reduction in total DNA synthesis and a mild reduction in the processivity of telomerase (Fig. 1A, 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\textsuperscript{2+} (as would be expected based on the primer/template alignment and the template sequence) (Fig. 1A, lanes 5 and 11), but by up to almost 20 nucleotides in the presence of Mn\textsuperscript{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\textsuperscript{2+}, but became much less active in the presence of Mn\textsuperscript{2+} (lanes 8–10). All of these observations are consistent with a significant loss of fidelity by telomerase in the presence of Mn\textsuperscript{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\textsuperscript{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\textsuperscript{2+}, we pretreated telomerase with RNase A and subjected the resulting preparation to primer extension analysis. As expected, in the presence of Mg\textsuperscript{2+} alone, the RNase pretreatment completely abolished the extension reaction (Fig. 2A, lanes 1 and 2). In contrast, in the presence of Mn\textsuperscript{2+} alone or Mn\textsuperscript{2+} and Mg\textsuperscript{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\textsuperscript{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. 2B, the ability of telomerase to add dCTP and dATP to the starting primer was indeed greatly enhanced by the addition of Mn\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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. 3A). Second, the TT activity was completely absent from a telomerase preparation in which two of the three catalytic
the presence of Mn$^{2+}$ from apparently conserved in evolution. For instance, the telomerase gene is deleted), and assayed in parallel. As shown in Fig. 3 both a wild-type and a important for the TT activity. Telomerase was isolated from our preparation may not have been completely removed by addressed the possibility that the telomerase RNA component in the TT activity is mediated by the active site of RT. We also greatly diminished TT activity (data not shown), suggesting 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. 1B), 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-
presence of labeled dCTP only. The reactions were performed in the presence of 2 mM Mg2+/H9251 polymerase as indicated above the panel. The labeled nucleotides included in the reactions are listed below the panel. (Left) incubated with or without 0.3 Lue et al. and plausible that the RNA can modulate protein conformation the covalent chemistry of telomerase (although it is entirely findings indicate that the entire RNA subunit is dispensable for RNA mediate important catalytic functions (7, 42–45). Our body of evidence, that nontemplate regions of the telomerase the RNA in catalysis. It has been argued, based on a substantial 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 Mn2+ 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 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.
shown schematically with the filled bars designating the locations of the TG-tracts. 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 Mn2+ 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.

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 Mn2+ 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 Mn2+ 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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