tein of comparable size (*24*). The accord that emerges from our calculations for basic thermodynamic parameters suggests that studies such as those presented here can be used for two purposes: (i) to provide a bridge to analytic mean-field and lattice-based theories, furnishing them with fundamental parameters for their model descriptions (*4*, *25*), and (ii) to provide predictions and rationalizations of experimental observations.

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13. Fragment B of staphylococcal protein A is a single-domain three–helix bundle protein that comprises 60 amino acids. The sequence of the 60-residue protein fragment is (key hydrophobic core residues are shown as bold) TADNKFNKEQQNA**F**YE**IL**H-**L**PN**L**NEEQRNG**FI**QS**L**KDDPSQS**A**NLL**A**E**A**KK**L**-NDAQAPKA. (Abbreviations for the amino acid residues are A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; and Y, Tyr.) In solution (*16*), the COOH-terminal helix III is intact and all three helices contact one another, forming a small (~30 side-chain contacts) hydrophobic core. In complex with an immunoglobulin (*15*), helix III is basically unfolded, even though it is not in direct contact with the protein, and helices I and II pack together, resulting in a significantly smaller interhelical angle and differing contacts. Our simulations are of the sequence from residues 10 to 55 (underlined), which includes the three helices and begins at the NH_2-terminal Gln^{10} and ends at the COOH-terminal Ala^{55}. In the simulations, the NH_2-terminus and COOH-terminus of the polypeptide were blocked with acetyl and *N*-methyl amine groups, respectively.
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17. To generate initial conditions spanning a relevant range of R_{gy}, six different MD simulations were performed. Each simulation consisted of a system defined by the protein plus 5012 water molecules in a periodic volume. The protein and solvent interacted via the CHARMM version 19 empirical force field [B. R. Brooks *et al.*, *J. Comput. Chem.* **4**, 187 (1983)], in which water is represented by the TIP3P model [W. L. Jorgensen, J. Chandrasekhar, J. Madura, M. L. Klein, *J. Chem. Phys.* **79**, 926 (1983)]. SHAKE was used to maintain hydrogen–heavy atom bond distances fixed and to render the water molecules rigid at their experimental geometry [J.-P. Ryckaert, G. Ciccotti, H. J. C. Berendsen, *J. Comput. Phys.* **23**, 327 (1977)]. Long-range forces were truncated at 10 Å with an atom-based force-shift method [C. L. Brooks III, B. M. Pettitt, M. Karplus, *J. Chem. Phys.* **83**, 5897 (1985)]. Interactions were computed with lists updated every 20 time steps [M. P. Allen and D. J. Tildesley, *Computer Simulation of Liquids* (Oxford University Press, Oxford, 1989); L. Verlet, *Phys. Rev.* **159**, 98 (1967)], and the time step for integration of the equations of motion was 2.0 fs. Two independent 1.3-ns simulations were performed on the "native" state at 300 K, starting from the NMR structure. The temperature was smoothly increased to 360 K and maintained there for another 800 ps. Each system was then heated to 400 K and held there for 800 ps. The trajectories were analyzed to extract a conformational database that spanned the range of R_{gy} from 9.3 to 12.5 Å. Sampling in large–protein volume regions was accomplished with four additional simulations initiated from structures, with both large and small R_{gy}, samples in the previous simulations. These structures were unfolded slowly and stepwise with a harmonic potential in R_{gy} centered around a value of 14 Å. In each instance, a small initial force constant was chosen and was increased as the simulation continued. After the structures reached the target value of R_{gy}, the biasing potential was removed and the structures were allowed to relax. Each of these simulations required between 300 and 500 ps to achieve the target value. These structures were added to the database. The database initially contained ~300,000 structures. Conformations that were nearest neighbors in time were eliminated to diminish correlation and reduce the database to a manageable size.
18. To extract the "natural" clustering from the hierarchy, we used the function

$$E(m)=\frac{MBCD(m) - MBCD(m+1)}{\sqrt{SSQ(m)} - \sqrt{SSQ(m+1)}}$$

which peaks strongly for clusterings that have compact, well-separated clusters (*19*). In this expression, *MBCD*(*m*) is the minimum between-cluster distance for clusters at level *m* of the hierarchy, and *SSQ*(*m*) is the mean sum of squared deviation between all structures at the *m*th level of the hierarchy. These clusters, and their representative centers, define the range of conformations sampled during folding. These structures form the basis for our analysis of the folding funnel shown in Fig. 1 and the folding pathway described in Fig. 2.
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Telomerase in Yeast

Marita Cohn and Elizabeth H. Blackburn*

The ribonucleoprotein enzyme telomerase synthesizes telomeric DNA by copying an internal RNA template sequence. The telomerase activities of the yeasts *Saccharomyces castellii* and *Saccharomyces cerevisiae*—with regular and irregular telomeric sequences, respectively—have now been identified and characterized. The *S. cerevisiae* activity required the telomerase RNA gene *TLC1* but not the *EST1* gene, both of which are required for normal telomere maintenance in vivo. This activity exhibited low processivity and produced no regularly repeated products. An inherently high stalling frequency of the *S. cerevisiae* telomerase may account for its in vitro properties and for the irregular telomeric sequences of this yeast.

Telomeres, the specialized DNA-protein structures at the ends of eukaryotic chromosomes, are necessary for chromosomal stability (*1*). The telomeric DNA that is essential for telomere function consists of simple sequences, repeated in tandem, whose synthesis and maintenance normally require the ribonucleoprotein enzyme telomerase (*2*–*5*). Telomerase activities identified in vitro in extracts from ciliated protozoan and vertebrate species catalyze the synthesis of homogeneous, precisely repeated telomeric sequences (*6*, *7*). Synthesis by telomerase occurs by addition of the G-rich telomeric strand to the 3′ end of a DNA primer. For the *Tetrahymena thermophila* telomerase, both in vitro and in vivo studies show that DNA synthesis occurs by copying a short template sequence within the RNA moiety of the enzyme (*2*, *8*).

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Telomerase activity in vitro has not been described for any yeast. The telomeric sequences of the yeast *Saccharomyces cerevisiae* are $TG_{2-3}(TG)_{1-6}$ repeats (*9*), and their irregularity has suggested that they might replicate by recombinational mechanisms (*10*). However, not all *Saccharomyces* species have irregular telomeric repeats: *S. kluyveri* telomeric sequences are homogeneous 26–base pair repeats, and *S. castellii* telomeric DNA consists of TCTGGGTG repeats (76%) interspersed with $TCTGGG(TG)_{2-4}$ repeats (24%) (*11*). We have now identified and characterized telomerase activities in *S. castellii* and *S. cerevisiae*. We reasoned that the relative regularity of the telomeric repeats of *S. castellii* would allow the detection of a periodic eight-base pattern synthesized by a telomerase from this species. We therefore used fractionated *S. castellii* extracts for the initial identification of conditions that would allow unambiguous detection of a budding yeast telomerase activity in vitro (*12*). The triphosphate substrates were [α-^{32}P]deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), and deoxycytidine triphosphate (dCTP). The initial primer used was the 16-nucleotide (nt) $(GTGTCTGG)_2$, consisting of the TG-rich strand of two *S. castellii* consensus telomeric repeats. Reaction products were analyzed on DNA sequencing gels. After optimizing conditions for the synthesis of long elongation products by the *S. castellii* polymerization activity, we used similar fractionation and reaction conditions to detect and then optimize activity in *S. cerevisiae* extracts (*12*).

The polymerization activities of both species fulfilled the essential criteria for telomerase activity (Fig. 1). Polymerization activity, revealed as a series of ^{32}P-labeled products longer than the input oligonucleotide primer, was dependent on input of the primer and was abolished by pretreatment of the extract with ribonuclease (RNase) A or proteinase K; the products were unaffected by RNase A treatment after the reaction (Fig. 1, A and B). Adding the RNase inhibitor RNasin to the extract before RNase A pretreatment allowed elongation products to be synthesized, which demonstrated that RNase A inhibited polymerization because of its enzymatic activity. Labeled high molecular mass products, migrating at limit mobility (Fig. 1), were not abolished by RNase A pretreatment and thus were not considered to be produced by telomerase activity. Telomerases from ciliates and vertebrates show specificity for G-rich, telomere-like primers in vitro (*6*, *7*, *13*), which reflects a complex combination of effects on different kinetic parameters of the in vitro reaction (*14*) and complementarity of the 3′ end of the primer with the RNA template sequence (*15*). The polymerization activities of *S. castellii* and *S. cerevisiae* shared the telomeric primer specificity characteristics of other known telomerases (*16*).

The single-copy telomerase RNA genes have been identified in two budding yeasts, *S. cerevisiae* (*TLC1* gene) and *Kluyveromyces lactis* (*TER1* gene) (*4*, *5*). In both species, disruption of the single-copy telomerase RNA gene causes progressive shortening of the telomeric DNA and eventual loss of cell viability (senescence). We investigated whether *TLC1* was essential for telomerase activity in vitro by preparing and assaying fractions in parallel from haploid *S. cerevisiae tlc1*-Δ strains, in which the *TLC1* coding sequence was deleted and replaced by a *LEU2* marker (*4*), and from the control haploid *TLC1*$^+$ strain sporulated from the diploid *TLC1*/*tlc1*-Δ strain (*4*). No telomerase activity was detectable in fractions from the *tlc1*-Δ cells (Fig. 1C). Addition of *tlc1*-Δ strain fractions to *TLC1*$^+$ strain fractions did not inhibit activity in the latter, which showed that the absence of activity in the strain without telomerase

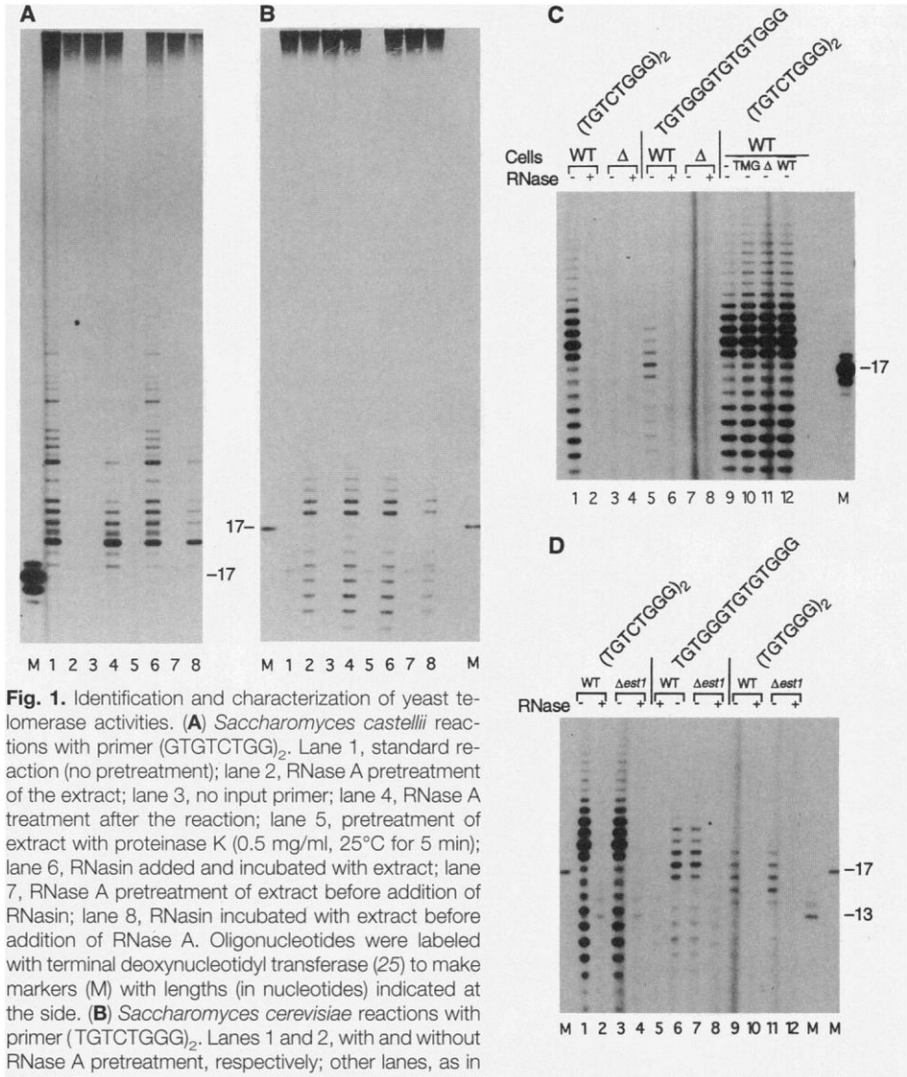

Fig. 1. Identification and characterization of yeast telomerase activities. (**A**) *Saccharomyces castellii* reactions with primer $(GTGTCTGG)_2$. Lane 1, standard reaction (no pretreatment); lane 2, RNase A pretreatment of the extract; lane 3, no input primer; lane 4, RNase A treatment after the reaction; lane 5, pretreatment of extract with proteinase K (0.5 mg/ml, 25°C for 5 min); lane 6, RNasin added and incubated with extract; lane 7, RNase A pretreatment of extract before addition of RNasin; lane 8, RNasin incubated with extract before addition of RNase A. Oligonucleotides were labeled with terminal deoxynucleotidyl transferase (*25*) to make markers (M) with lengths (in nucleotides) indicated at the side. (**B**) *Saccharomyces cerevisiae* reactions with primer $(TGTCTGGG)_2$. Lanes 1 and 2, with and without RNase A pretreatment, respectively; other lanes, as in (A). (**C**) Telomerase analysis of fractions from haploid *S. cerevisiae* strains with a wild-type *TLC1* gene (WT) (lanes 1, 2, 5, and 6) or a disrupted *tlc1* gene (Δ) (lanes 3, 4, 7, and 8) sporulated from diploid *TLC1*/*tlc1*-Δ strain UCC3508 (*4*). The *tlc1* and wild-type extracts were prepared identically and corresponding fractions from the DEAE-agarose column were assayed for telomerase activity under identical conditions. Lanes 9 to 12: the wild-type strain YPH399 (*24*) fractions used in (B) with no addition (lane 9) or mixed with equal volumes of TMG buffer (lane 10) or *tlc1*-Δ (lane 11) or wild-type (lane 12) haploid strain fractions. Primers and RNase pretreatment (+ or −) were as indicated. (**D**) Telomerase analysis of extract from a haploid *S. cerevisiae* strain with a disrupted *EST1* gene (Δ*est1*) (*26*). The *est1* extract was prepared identically to the *S. cerevisiae* wild-type extract and the activity eluted in the same fraction from the DEAE-agarose column. Wild-type extract (WT) (lanes 1, 2, 5, 6, 9, and 10) and *est1* extract (lanes 3, 4, 7, 8, 11, and 12) were assayed for telomerase activity under identical conditions with primers and RNase pretreatment (+ or −) as indicated.

RNA was not attributable to a trans-acting negative inhibitor (Fig. 1C). This result confirmed that the *S. cerevisiae* polymerization activity is telomerase activity.

Normal telomere maintenance in *S. cerevisiae* requires the *EST1* gene, and disruption of *EST1* causes telomere shortening and senescence (*17, 18*). Because these phenotypes are expected to result from a loss of telomerase activity, and because *EST1* encodes a ~70-kD protein that contains consensus motifs shared by reverse transcriptases (*19*), it was suggested that *EST1* may encode a telomerase component (*17*). However, in contrast to the *tlc1*$^-$ deletion strains, in vitro telomerase activity of the haploid *S. cerevisiae* strain *est1-Δ3*, in which 80% of the *EST1* coding sequence is deleted and replaced by a *HIS3* marker (*17*), was identical to that of the *EST1*$^+$ haploid control with respect to level of activity, product profiles with three different primers at two different temperatures, and RNase sensitivity (Fig. 1D) (*11*). Therefore, *EST1* does not encode a protein that is indispensable for the catalytic activity of telomerase or for binding to the primer DNA in vitro. The EST1 protein may be required to augment telomeric DNA binding or to recruit telomerase to the telomere through protein-protein interactions (activities that were not assayed here). Alternatively, EST1 may play a different role in telomere maintenance, such as protection of the telomeric DNA.

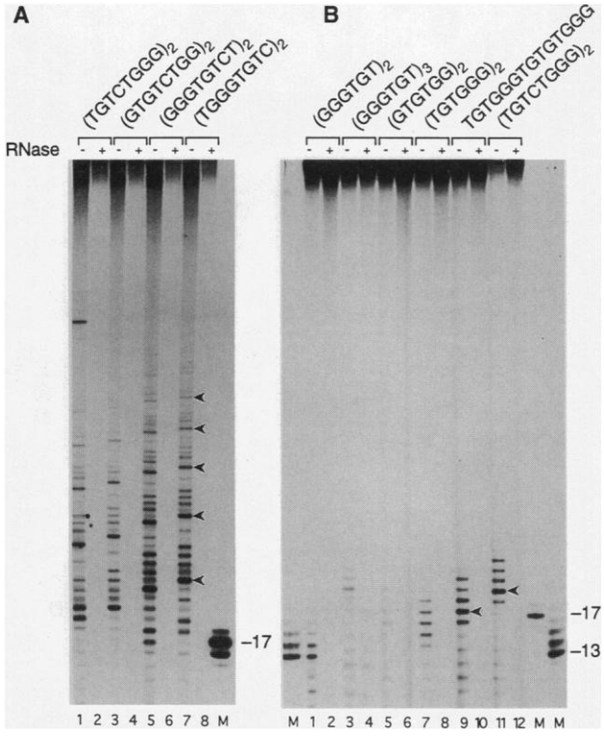

Fig. 2. Analysis of template-directed synthesis. (**A**) *Saccharomyces castellii* reactions with primers corresponding to different permutations of the *S. castellii* telomeric sequence $(TGTCTGGG)_2$. Arrowheads indicate the most intense band in the repeated pattern. (**B**) *Saccharomyces cerevisiae* reactions with different permutations of *S. cerevisiae* telomeric primers as indicated. Samples in lanes 11 and 12 corresponded to reaction volumes of 20 μl; the other samples had reaction volumes of 40 μl. M, markers for 17 and 13 nt as indicated (*25*). RNase A pretreatment (+ or −) was as indicated.

Each yeast polymerization activity behaved as would be expected if it were directed by a telomerase RNA template. Although telomerase RNA from *S. castellii* has not been isolated, the octameric TCTGGGTG telomeric repeats were expected to be copied from an RNA template region that includes at least one permutation of a full repeat unit. The moderately processive *S. castellii* telomerase activity typically produced five to seven distinct 8-nt repeats (Fig. 2A). With circularly permuted 16-nt *S. castellii* telomeric primers, the banding patterns shifted as predicted for template-directed addition onto the different 3′ ends of these primers (Fig. 2A): one step upward between lanes 1 and 3, two steps upward between lanes 3 and 5, and one step upward between lanes 5 and 7. The most intense band in the periodic pattern corresponded to addition of the first T residue in the *S. castellii* sequence TCTGGGTG. For example, with a primer ending -TCTGG, the first strong dissociation band corresponded to addition of the fourth nucleotide, with an 8-nt repeating pattern extending above it (Fig. 2A). As shown for *Tetrahymena* telomerase, this strongest band may correspond to dissociation after copying the 5′ end of the internal template in the *S. castellii* telomerase RNA. We propose that tandem CTGGGTGT repeats are synthesized with a minimal hypothetical nine-base template region, 3′-AGACCCACA-5′ (Fig. 3A).

The irregular telomeric repeats in *S. cerevisiae* consist of ~50% TGGGTG repeats; the remainder consist of TGGG or TGG followed by one to six TG dinucleotides (9). The longest possible telomerase RNA template sequence in *TLC1* contains 16 bases that could direct synthesis of these irregular repeats (*4*). The *S. cerevisiae* activity extended TGGGTG repeat primers by ~5 to 15 nt, with no visible periodicity. However, with two primers of different lengths (12 or 18 nt) but with the same -TGT 3′ end, the two darkest bands were shifted up as predicted for template-directed synthesis (Fig. 2B). Likewise, with two primers with -TGGG 3′ ends, the banding patterns were similar, with that of the primer that was 2 nt

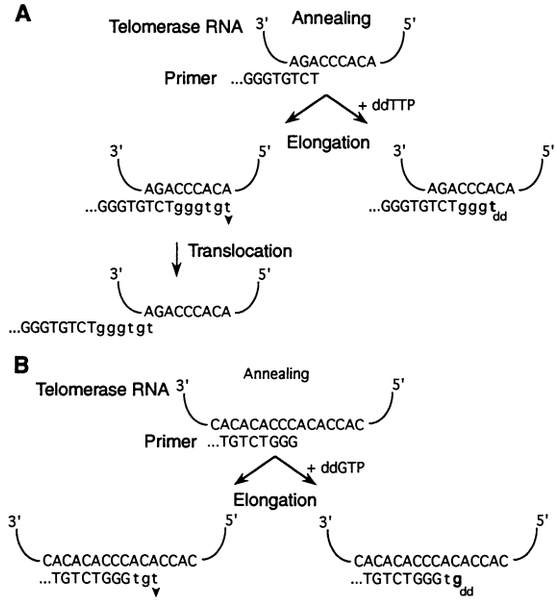

Fig. 3. Schematic model of yeast telomere elongation. (**A**) A hypothetical minimal telomerase RNA template region is proposed for *S. castellii* based on the observed pausing pattern. The three steps of telomerase action are shown: (i) annealing of the primer $(GGGTGTCT)_2$ to the 3′ end of the template RNA, (ii) elongation of the primer by copying the RNA to the 5′ end of the template region, and (iii) translocation of the newly elongated strand to the 3′ end of the template RNA to allow a second round of synthesis. The predicted premature termination position when ddTTP is present in the reaction mixture is indicated ($\mathbf{t}_{dd}$). (**B**) The predicted annealing of the $(TGTCTGGG)_2$ primer to the *S. cerevisiae* telomerase RNA template (*4*) with subsequent elongation; the predicted premature termination position in the presence of ddGTP is indicated ($\mathbf{g}_{dd}$). Arrowheads in (A) and (B) indicate the observed major dissociation-pausing positions.

longer shifted up by two positions (Fig. 2B). These two -TGGG primers did not have the same base composition; hence, as expected, their patterns were slightly staggered. With the primers ending in -TGGG, which are expected to pair to the template as in Fig. 3B, the most intense band was at the third addition position (Fig. 2B, arrowheads). Thus, elongation may often extend only to the template position shown in Fig. 3B (downward arrowhead), a position that aligns with the 5′ end we propose for the telomerase RNA template in *S. castellii* (Fig. 3A).

Substituting one dideoxynucleoside triphosphate (ddNTP) for its dNTP analog in the reactions directly confirmed the schemes for template-directed synthesis shown in Fig. 3. When the *S. castellii* activity was primed by an oligonucleotide ending in -TCT, ddTTP terminated the synthesis after addition of 4 nt, as predicted (Fig. 4A, lane 8). With primers ending in -GTCT or -GTC, ddCTP caused the predicted strong stop after adding 7 or 8 nt, respectively (Fig. 4A; compare lanes 6 and 7 with control lanes 4 and 5). These ddNTP experiments also suggest how the mode of action of the *S. castellii* telomerase can account for the specific, limited irregularity of *S. castellii* telomeric repeats, in which perfect octameric TCTGGGTG repeats are interspersed with repeats containing one to three extra TG dinucleotides (*11*). In Fig. 4A, a doublet band was produced at the fourth and fifth elongation positions in lane 12 (-TGGG primer) and lane 2 (-TGG primer), respectively. For each doublet, although the downward shift of the lower band indicated incorporation of the predicted ddC residue (*20*), the higher band showed that another residue in addition to ddC was also incorporated at this position. This failure to terminate chain synthesis was also indicated by the presence of weak bands above the doublet. Lanes 6 and 7 (Fig. 4A) most clearly show two weak bands above the strong ddC stop. This production of doublet bands and elongation beyond the ddNTP position was specific to the ddCTP reactions and is likely to be attributable to addition of an extra TG dinucleotide. The results can be explained if the enzyme specifically "stutters" after synthesizing -TGGGTG by translocating back two positions on the template, thus extending the -TGGGTG sequence with another TG before adding TC. Such specific stuttering may be an intrinsic property of the *S. castellii* telomerase and may account for the variable number of TG dinucleotides in vivo. For the *S. cerevisiae* reaction, as predicted by the template-directed elongation scheme (Fig. 3B), with a primer ending in -TGGG and in the presence of [α-^{32}P]dTTP and ddGTP, one labeled dT residue was incorporated in the first position and a terminating ddG in the second (Fig. 4B, lanes 4, 8, and 12; arrowheads). Use of [α-^{32}P]dGTP and ddTTP blocked elongation of the same primer (Fig. 4B).

In *Tetrahymena*, a telomerase-based nucleolytic activity can remove a nucleotide from the 3′ end of a primer or product (*21*). With the yeast activities, especially that of *S. cerevisiae*, labeled products were often shorter than the input primer by up to 8 nt. Several lines of evidence indicate that these shortened labeled products are produced by a cleavage activity associated with yeast telomerase and not by a separate contaminating nuclease (*22*). The yeast telomerase-associated nucleolytic cleavage activity, like that of *Tetrahymena* telomerase (*21*), appears to act specifically on reaction products still bound to telomerase, but it seems capable of more extensive shortening.

The telomerase-based nucleolytic cleavage activity was particularly marked for the *S. cerevisiae* telomerase, which was also much less processive than the *S. castellii* activity. Mixing *S. cerevisiae* and *S. castellii* active fractions did not decrease the *S. castellii* processivity (*11*), which suggested that low processivity is intrinsic to *S. cerevisiae* telomerase rather than caused by inhibitors in the *S. cerevisiae* fractions. An inherently high frequency of stalling by the *S. cerevisiae* telomerase probably underlies both its high frequency of product dissociation at several positions along the RNA template sequence and its high cleavage activity. The 3′ end of the DNA primer can potentially be repositioned for elongation at alternative positions on the 16-base partially repeated RNA template of *S. cerevisiae* telomerase (Fig. 3B) (*4*). A high stalling frequency, directly resulting in product dissociation and cleavage, will create frequent opportunities for such alternative positioning along the template and hence for synthesis of irregular repeats. High nucleolytic cleavage activity of the *S. cerevisiae* telomerase may also account for the telomere shortening observed in vivo when the steady-state concentration of telomerase RNA, and thus potentially of telomerase ribonucleoprotein, is increased by overexpression of *TLC1* (*4*).

The yeast *S. cerevisiae* is a valuable system for molecular genetic studies of telomere function (*23*). Biochemical studies of telomerase enzyme function are now possible. The combination of observations on the mechanistic properties of telomerase activity in vitro with genetic approaches should provide an integrated view of yeast telomere functions.

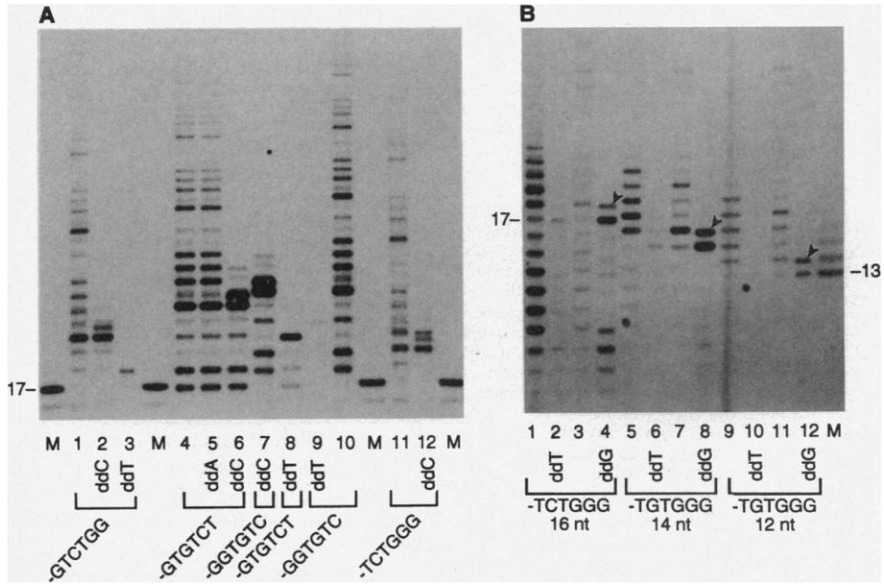

Fig. 4. Template-directed termination of telomerase-mediated DNA synthesis with ddNTPs. One of the dNTPs was replaced with the same concentration (50 μM) of the ddNTP analog in telomerase reactions. (**A**) *Saccharomyces castellii* reactions. Lanes 1 to 3: primer $(GTGTCTGG)_2$ with the standard reaction (lane 1), ddCTP (lane 2), or ddTTP (lane 3). Lanes 4 to 6 and 8: primer $(GGGTGTCT)_2$ with the standard reaction (lane 4), ddATP (50 μM ddATP was added to the regular reaction conditions) (lane 5), ddCTP (lane 6), or ddTTP (lane 8). Lanes 7, 9, and 10: primer $(TGGGTGTC)_2$ with ddCTP (lane 7), ddTTP (lane 9), or the standard reaction (lane 10). Lanes 11 and 12: primer $(TGTCTGGG)_2$ with the standard reaction (lane 11) or ddCTP (lane 12). (**B**) *Saccharomyces cerevisiae* reactions. Lanes 1 to 4, primer $(TGTCTGGG)_2$; lanes 5 to 8, primer TGTGGGTGTGTGGG; lanes 9 to 12, primer $(TGTGGG)_2$. Lanes 1, 5, and 9, standard reaction with [α-^{32}P]dGTP and dTTP; lanes 2, 6, and 10, [α-^{32}P]dGTP and ddTTP; lanes 3, 7, and 11, standard reaction with [α-^{32}P]dTTP and dGTP; lanes 4, 8, and 12, [α-^{32}P]dTTP and ddGTP.

Note added in proof: After this report was accepted for publication, a paper (*27*) concluded from a different assay that the EST1 protein is required for telomerase activity. Our results show that EST1 is neither an essential catalytic nor primer-binding component of telomerase.

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12. Whole cell extracts were prepared as follows: Cells (*24*) harvested in early log phase (optical density at 600 nm = 1) were resuspended in TMG buffer [10 mM tris-HCl (pH 8), 1.2 mM $MgCl_2$, 15% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM dithiothreitol, 1 mM pefabloc, 1 μM pepstatin, 1 μM leupeptin, 130 μM bestatin, and RNasin (40 units/ml)] and disrupted in a Bead-Beater (Biospec Products). S-100 extracts were prepared by ultracentrifugation at 100,000*g* for 90 min at 4°C, divided into portions, and stored at −80°C; the protein concentration was 22 mg/ml for *S. castellii* and 17 mg/ml for *S. cerevisae* S-100 extracts. The S-100 supernatants were fractionated on a DEAE-agarose column (Biogel; Bio-Rad) that had been equilibrated with TMG buffer (with 10% glycerol). The column was washed with four volumes of TMG buffer containing 0.5 M sodium acetate for *S. castellii* and 0.6 M sodium acetate for *S. cerevisiae*. After elution with 1.8 column volumes of 0.65 or 0.75 M sodium acetate in TMG buffer for *S. castellii* and *S. cerevisiae*, respectively, eluted fractions were desalted and concentrated on Microcon-30 columns (Amicon). Protein concentrations were not measured after purification. It was anticipated that the same relative amounts were still present in the extracts because they were treated identically. *Saccharomyces cerevisiae* extracts were diluted ~100 times and *S. castellii* extracts ~10 times during the Microcon-30 column desalting. For telomerase assays, equal volumes of desalted DEAE fractions (usually 10 or 20 μl) and 2× reaction buffer were mixed and incubated at either 20° or 30°C for 30 min. Final concentrations for *S. castellii* were 50 mM tris-HCl (pH 8), 1 mM spermidine, 1 mM dithiothreitol, 100 mM potassium glutamate, 50 μM dCTP, 50 μM dTTP, 1.9 μM [α-^{32}P]dGTP (800 Ci/mmol), and 1 μM primer (*25*). Potassium glutamate and dCTP were omitted for *S. cerevisiae* reactions. Reaction products were resolved on denaturing 10% acrylamide gels containing 7 M urea as described (*14*). RNase treatment of extracts was performed with RNase A (6.3 μg/ml) at 25°C for 5 min, immediately before starting the reaction. RNase A was inhibited by incubating the extract with RNasin (2300 units/ml) at 25°C for 5 min before addition of RNase A (6.3 μg/ml) and incubation for another 5 min.
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16. *Saccharomyces castellii* telomerase activity was also primed with 12- to 18-nt *S. cerevisiae* or *Tetrahymena* telomeric repeats. The *S. cerevisiae* activity was primed efficiently by oligonucleotides consisting of the *Tetrahymena* telomeric sequence $(T_2G_4)_3$, or by permutations of the *S. castellii* telomeric repeats with T or G, but not C, residues at the 3′ end. Non-telomeric oligonucleotides did not prime synthesis by either extract (*11*).
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22. Shortened labeled reaction products were not produced by elongation of degraded input primer, as shown by 5′ end labeling of the bulk primer (present in excess over telomerase) after it had been subjected to the telomerase reaction; >95% of the bulk primer remained full length. The short labeled products were not formed by initial telomerase elongation and subsequent degradation of the labeled elongation products by a contaminating nuclease as shown by pulse-chase experiments, chased with either ddGTP or excess unlabeled dGTP, in the presence or absence of RNase. During each chase there was no further production of shorter labeled products or breakdown of the products longer than input size. Moreover, the nucleolytic cleavage activity coeluted with the telomerase elongation activity in step elutions from DEAE-agarose, heparin-agarose, and octyl-Sepharose (Pharmacia) columns (*11*).
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24. Cell strains were *S. castellii* NRRL Y-12630 and *S. cerevisiae* YPH399 (*MAT*α, *ura3-52*, *lys2-801*, *ade2-101*, *his3* Δ*200*, *leu2-*Δ*1*, *trp1-*Δ*63*, *gal2*$^{+/-}$).
25. All oligonucleotides were purified to a single species by separation on a denaturing gel. Markers were created by elongation of primers $(TGTGGG)_2$ and $(GGGTGTCT)_2$ with terminal deoxynucleotidyl transferase (Boehringer Mannheim) and [α-^{32}P]dGTP. The primary products were 13 and 17 nt, respectively.
26. The diploid *S. cerevisiae* strain TVL115 contains the *est1-*Δ*3::HIS3* mutation, in which ~80% of the *EST1* gene is deleted. The heterozygous diploid was sporulated and the tetrads dissected. The haploid deletion strain showed the expected senescence and correct genomic band on Southern (DNA) blot analysis (*11*) with an *EST1* riboprobe (*17*). The protein concentration of the *est1* S-100 extract was 16 mg/ml.
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Simultaneous Identification of Bacterial Virulence Genes by Negative Selection

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An insertional mutagenesis system that uses transposons carrying unique DNA sequence tags was developed for the isolation of bacterial virulence genes. The tags from a mixed population of bacterial mutants representing the inoculum and bacteria recovered from infected hosts were detected by amplification, radiolabeling, and hybridization analysis. When applied to a murine model of typhoid fever caused by *Salmonella typhimurium*, mutants with attenuated virulence were revealed by use of tags that were present in the inoculum but not in bacteria recovered from infected mice. This approach resulted in the identification of new virulence genes, some of which are related to, but functionally distinct from, the *inv/spa* family of *S. typhimurium*.

Several different approaches have been used to exploit transposon mutagenesis for the isolation of bacterial virulence genes, including screens for the loss of specific virulence-associated factors (*1*), survival within macrophages (*2*), and penetration of epithelial cells (*3*). Although these screens have identified many bacterial genes required for virulence, they are restricted to certain stages of infection. Transposon mutants have also been tested individually for altered virulence in live animal models of infection (*4*), but comprehensive screening of bacterial genomes for virulence genes has not been possible because of the inability to identify mutants with attenuated virulence within pools of mutagenized bacteria and the impracticability of separately assessing the virulence of each of the several thousand mutants necessary to screen a bacterial genome. We have circumvented this problem by developing a transposon mutagenesis system, termed signature-tagged mutagenesis, in which each transposon mutant

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