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Association of the Est1 protein with telomerase activity in yeast

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ABSTRACT **The *est1* mutant was previously identified because it is defective in telomere maintenance and displays a senescent phenotype. To see if Est1 might be a component of yeast telomerase, we examined immunoprecipitated Est1. The yeast telomerase RNA Tlc1 specifically coprecipitated with Est1. Furthermore, the Est1 immunoprecipitates contained a telomerase-like activity. As expected for yeast telomerase, the activity elongated telomeric primers, it required dGTP and dTTP but not dATP or dCTP, and it was sensitive to RNase A. Further evidence suggesting that the activity was telomerase was obtained from experiments using a *TLC1-1* mutant strain, which has a mutant telomerase template containing dG residues. The activity immunoprecipitated from *TLC1-1* mutant strains incorporated ^{32}P-labeled dCTP, while activity from *TLC1* strains did not. Use of different telomeric primer substrates revealed two distinguishable telomerase-like activities: one was dependent on *TLC1*, and one was not. The *TLC1*-independent activity may be due to a second yeast telomerase RNA, or it may be some other kind of activity.**

The ends of eukaryotic chromosomes are capped by structures called telomeres which prevent the ends from being degraded (1, 2). Telomeric DNA consists of repeats of simple sequences, such as TTAGGG in humans, and TG_{1-3} in the yeast *Saccharomyces cerevisiae.* Telomeres are maintained by an enzyme called telomerase, which adds the repeats to the very ends of the chromosomes, thereby balancing the loss of repeats which occurs during replication (3–5). Telomerase is a remarkable enzyme containing both RNA and protein components. The RNA component contains the template for addition of repeats. For instance, the *Tetrahymena thermophila* telomerase RNA contains the sequence CAACCCCAA, which templates the addition of TTGGGG repeats (6). The yeast telomerase RNA Tlc1 contains the sequence ACCACACCCACACAC, which templates the addition of the TG_{1-3} repeats (7). Since mature yeast telomeres contain mostly imperfect copies of the template (8, 9), copying in yeast must be degenerate and imprecise.

Telomerase activity has been discovered in a number of organisms, and the telomerase RNA has been cloned in a few cases (3). Two protein components of telomerase have recently been identified in Tetrahymena; these show little homology to other known proteins (10). One possible candidate for a yeast telomerase component is the Est1 (Ever Shorter Telomeres) protein (11). Yeast cells lacking *EST1* suffer telomere shortening, and clonal cultures undergo senescence, with most cells in the culture dying after 80–100 population doublings (11, 12).

MATERIALS AND METHODS

Yeast Media, Strains, and Plasmids. Standard methods were used for growth and manipulation of yeast (13). For telomerase assays, yeast were grown in 1% yeast extract/2% peptone/2% dextrose (YEPD). All strains were isogenic with W303α [*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100 ssd1-d* (*psi*$^+$)] (14). The genomic copy of *EST1* was tagged at the 5′ end with a triplicated hemagglutinin tag (15) by a recombinational method (16). The *TLC1-1* mutant strain was created by transformation of cells with plasmid pRS306-TLC1-1 (*Hae* III). The *tlc1* deletion/disruption strain was created by transformation with the *Xho* I fragment of plasmid pBlue61::LEU2. These plasmids were kindly provided by M. Singer and D. Gottschling (7). The *est1* disruption was made by inserting *URA3* after the start codon by a recombinational method (17). This disruption allele had an Est$^-$ phenotype, but we have no evidence that it is a null allele.

Yeast Extracts and Immunoprecipitation. Yeast extracts were made by beating yeast cells with zirconia beads (0.5-mm diameter; Biospec Products) and buffer A [50 mM Tris-acetate, pH 7.6/50 mM potassium acetate/0.2 mM EDTA/0.2 mM EGTA/5% (vol/vol) glycerol/4 mM dithiothreitol/100 μg of aprotinin per ml/4 μg of pepstatin per ml/4 μg of leupeptin per ml/100 μg of phenylmethylsulfonyl fluoride per ml/200 μg of soybean trypsin inhibitor per ml/100 μg of 7-amino-1-chloro-3-tosylamido-2-heptanone (TLCK) per ml/10 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) per ml/10 units of RNase inhibitor per ml] for 50 sec in a mini-bead beater (Biospec Products) at medium-high speed. The extract was clarified by two centrifugation steps at 16,000 × *g* for 10 min and 15 min, respectively. The protein concentration was determined by the Bradford assay, with reagent from Bio-Rad.

For immunoprecipitation for telomerase assays, 0.4 μl of ascites fluid from the 12CA5 cell line was added for each 5 mg of protein in the sample of extract. The solution was incubated on ice for 1.5 h. Washed protein A beads (Pierce) were added (5 μl of beads per 5 mg of protein), and the mixture was incubated with gentle agitation for 2 h at 4°C. The protein A beads were collected by a very brief, very gentle centrifugation (1 sec, <500 × *g*) and washed four times with buffer B [25 mM Tris-acetate, pH 8.2/50 mM potassium acetate/1 mM EGTA/1 mM $MgCl_2$/10% (vol/vol) glycerol/2 mM dithiothreitol] supplemented with 5 units of RNase inhibitor per ml. Centrifugation steps were brief (1 sec or less) and used minimal *g* forces; otherwise, various activities were precipitated non-specifically. Finally, the washed beads were resuspended. For every 5 mg of protein in the original sample of extract, 20 μl of buffer B plus 0.1 units of RNase inhibitor was added.

Coimmunoprecipitation of Tlc1 with Est1 was similar, but a high-salt lysis buffer was used (50 mM Tris·HCl, pH 7.5/250 mM NaCl/50 mM NaF/5 mM EDTA/0.1% Nonidet P-40/5 mM dithiothreitol. Cell extracts were incubated with antibody for 20 min. Extract containing 10 mg of protein was immunoprecipitated by using 0.6 μl of 12CA5 ascites fluid, and one-eighth of the immunoprecipitate was loaded on the gel. Epitope peptide competition experiments used 0.6 μl of 12CA5 ascites fluid, 100 μg of competitor peptide (YPYDVPDYA), and extract containing 10 mg of protein. In Fig. 1, lanes 1 and 4 contain the total RNA from extract containing 12.5 μg of protein—i.e., they represent about 1% as much extract as the lanes containing immunoprecipitates.

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Est1 protein could not be seen by direct Western analysis when 50 μg of total yeast protein was loaded on the gel. However, Est1 could be seen on a Western blot after it was immunoprecipitated from extract containing 4 mg of total yeast protein (16).

Telomerase Assays. Telomerase assays were performed in a volume of 40 μl in buffer B without glycerol with 20 μl of immunoprecipitate (corresponding to 5 mg of initial protein). Further additions were 1 μM dGTP (of which 0.25–0.5 μM was [α-^{32}P]dGTP; 3000 Ci/mmol; 1 Ci = 37 GBq, 100 μM dTTP, 1 mM spermidine, and 2 μM substrate oligonucleotide. The reaction was incubated at 30°C. After 2 h., 160 μl of water was added, and the mixture was extracted with 200 μl of phenol/chloroform/isoamyl alcohol (25:24:1). Ammonium acetate and $MgCl_2$ were added to the aqueous phase to concentrations of 2.5 M and 10 mM, respectively, 10 μg of yeast tRNA was added, and nucleic acids were precipitated with two volumes of cold ethanol. The DNA was pelleted by centrifugation, washed twice with cold 70% (vol/vol) ethanol, dried, and loaded onto a 12% acrylamide/7M urea sequencing gel. Gels were dried and placed on Fuji PhosphorImager screens for about two days. RNase A sensitivity was shown by incubating the immunoprecipitates with 10–100 ng of heat-treated RNase A for 20 min at room temperature before addition of other assay components. Telomerase activity was not destroyed if a mixture of 100 ng of RNase A and 160 units of human placental RNase inhibitor (Boehringer Mannheim) were added to the immunoprecipitate. Oxidized, inactive RNase A was obtained from Sigma.

RESULTS

To see if Est1 might be a component of telomerase, we tagged Est1 at its N terminus with three tandem copies of the hemagglutinin epitope. The tagged gene complemented an *est1* mutant, although the phenotype was not completely wild type. We were able to detect the tagged Est1 on immunoblots, but only after it had been concentrated by immunoprecipitation (data not shown).

When the tagged Est1 protein was immunoprecipitated, the telomerase RNA was coprecipitated (Fig. 1). Little or no telomerase RNA was precipitated from an untagged strain by the same antibodies, and addition of competing epitope peptide blocked coimmunoprecipitation, showing that the association of Tlc1 with Est1 was specific. Only 1–5% of the Tlc1 RNA in the extracts could be coprecipitated with Est1. Since we cannot see Est1 by direct Western analysis (*Materials and Methods*), we do not know how efficiently Est1 is immunoprecipitated. Therefore, we do not know whether the low yield of Tlc1 is because only a small portion of the Tlc1 in the cell is associated with Est1 or because Est1 is inefficiently immunoprecipitated.

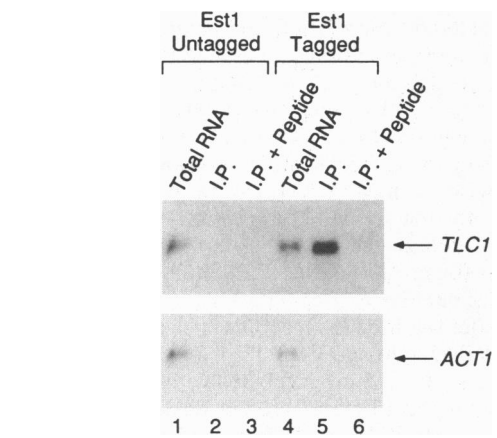

FIG. 1. Tlc1 coprecipitates with Est1. Northern blot of RNA from an untagged yeast strain (lanes 1, 2, and 3) or from a strain carrying tagged Est1 (lanes 4, 5, and 6). Lanes 1 and 4 show total RNA from the two strains. Lanes 2 and 5 show the RNAs in the 12CA5 immunoprecipitates (I.P.s). Lanes 3 and 6 show the RNAs in the immunoprecipitates in an experiment where a synthetic epitope peptide was added to block the antibody. The blot was hybridized to a *TLC1* probe and to an *ACT1* (actin) probe as a loading control. Lanes 2, 3, 5, and 6 (immunoprecipitates) represent about 100 times more extract than lanes 1 and 4 (total RNA) (see *Materials and Methods*).

A telomerase-like activity was found in the Est1 immunoprecipitates. When incubated with a suitable substrate oligonucleotide, dTTP, [α-^{32}P]dGTP, and an appropriate buffer, about 20 nt were added to the substrate oligonucleotide (Fig. 2). Optimal activity required the simultaneous presence of both dTTP and dGTP (Fig. 3) but not dCTP or dATP. *Tetrahymena* and human telomerase give a distinctive, phased pattern of strong and weak bands; no such pattern was seen here. However, yeast telomeres contain degenerate copies of the telomerase template, perhaps suggesting that the enzyme slips frequently, so a phased pattern is not necessarily expected.

As expected for telomerase, the activity was destroyed by as little as 10 ng of RNase A (Fig. 2). An inactive, oxidized form of RNase A failed to inhibit the telomerase-like activity (data not shown). Furthermore, a mixture of RNase A and RNase inhibitor failed to inhibit the telomerase-like activity (data not shown). Thus, the telomerase-like activity was sensitive to ribonuclease.

We considered the possibility that some reverse transcriptase might be transcribing RNA molecules fortuitously present in the immunoprecipitates. To test this, we treated an immunoprecipitate with RNase A, then added RNase inhib-

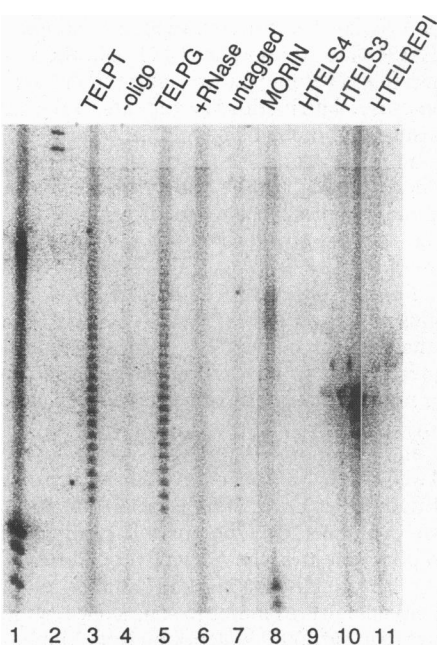

FIG. 2. Elongation of oligonucleotides by the telomerase-like activity. Telomerase assays were done on a variety of oligonucleotides by using 12CA5 immunoprecipitates from a tagged Est1 strain. Reactions contained dTTP and [^{32}P]dGTP. TELPT, TELPG, MORIN, etc. are the names of substrate oligonucleotides; the sequences are presented in Table 1. Lanes 4, 6, and 7 were controls. Lane 4, no added oligonucleotide (−oligo); lane 6, immunoprecipitate treated with 10 ng of RNase A before telomerase assay with oligonucleotide TELPT (+RNase); and lane 7, extract from an untagged strain assayed with oligonucleotide TELPT (untagged). Lane 1 shows end-labeled oligonucleotide TELPG, and lane 2 shows end-labeled restriction fragments of about 75 bp, as size markers.

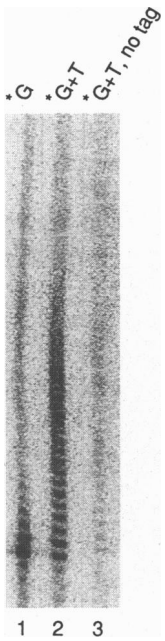

FIG. 3. Optimal telomerase-like activity requires both dGTP and dTTP. Telomerase assays were carried out with immunoprecipitates from a tagged Est1 strain (lanes 1 and 2) or an untagged strain (lane 3) by using TELPT as the substrate oligonucleotide. In lane 1 [^{32}P]dGTP was added but not dTTP (similar results were obtained with [^{32}P]dTTP alone; data not shown). In lanes 2 and 3, [^{32}P]dGTP and unlabeled dTTP were added.

itor, and finally added back 0.1, 1, or 10 μg of total yeast RNA. Despite the readdition of RNA, no telomerase-like activity was detected (data not shown). [Reconstitution of *bona fide* telomerase would not be expected because the amount of Tlc1 RNA in the total yeast RNA was very small. Reconstitution of *Tetrahymena* telomerase is inefficient even with semipurified proteins and large amounts of pure telomerase RNA (18).] Thus, the RNase A sensitivity was not due to a nonspecific requirement for RNA.

The activity was quite specific for oligonucleotides mimicking the G-rich strand of yeast telomeres (Table 1). Several oligonucleotides modeled on the yeast telomere were assayed, and all were elongated. In contrast, oligonucleotides modeled on the human telomere or on the C-rich strand of the yeast telomere were not elongated. Several other test oligonucleotides of unrelated sequence were also assayed. Some of these incorporated a few nucleotides, but none gave the 20-nt ladder typical of the yeast telomeric primers.

To provide further evidence that the activity in the Est1 immunoprecipitates was yeast telomerase, we took advantage of the *TLC1-1* mutation (7). The template region of wild-type Tlc1 RNA contains no G residues, but in the mutant Tlc1-1 RNA, a C residue and the adjacent A residue have been changed to G residues. Since the mutant telomerase has G residues in the template, it now incorporates dCTP *in vivo* (7). We compared Est1 immunoprecipitates from a *TLC1* strain and a *TLC1-1* mutant strain. When given dTTP, dCTP, and [α-^{32}P]dGTP, the activities from the two strains used the [α-^{32}P]dGTP equally well. However, when given dTTP, dGTP, and [α-^{32}P]dCTP, the activity precipitated from *TLC1* strains incorporated very little or no [α-^{32}P]dCTP. Activities precipitated from the *TLC1-1* strains were variable: in some cases (Fig. 4), there was very good incorporation of [α-^{32}P]dCTP, while in other cases (data not shown) the incorporation was not better than for the *TLC1* experiment.

One possible explanation for the variability of [α-^{32}P]dCTP incorporation with extracts from the *TLC1-1* strain is that only a part of the template region is used, and the mutant region is not always copied. We tested this possibility using oligonucleotide TELPGGG, which ends in GGG (Table 1) (an experiment suggested by D. Gottschling). This oligonucleotide can align at only one place along the template, immediately adjacent to the mutant nucleotides. When this oligonucleotide was used in telomerase reactions, incorporation of [α-^{32}P]dCTP was robust and repeatable with extracts from the *TLC1-1* strain, but there was little or no incorporation with extracts from the *TLC1* strain (Fig. 5). This shows that the activity seen with oligonucleotide TELPGGG was being templated by *TLC1-1*.

We also assayed activity in Est1 immunoprecipitates from a *tlc1* deletion strain. The *tlc1* deletion strain had a senescent phenotype. Southern analysis showed that no *TLC1* gene was present, and Northern analysis showed that no Tlc1 RNA was present (data not shown). When oligonucleotide TELPGGG was used as the substrate, the *tlc1* extracts showed no telomerase activity, as expected (Fig. 6). However, surprisingly, some telomerase-like activity was seen in *tlc1* extracts when oligonucleotide TELPT was used (Fig. 6). The activity seen with oligonucleotide TELPT was still sensitive to RNase A (Fig. 6). Thus, it appears that there are at least two activities in the Est1 immunoprecipitates: one which can elongate oligonucleotide TELPGGG (and probably TELPT as well) and is *TLC1* dependent, and one which cannot elongate TELPGGG and is not *TLC1* dependent. Both these activities require dGTP and dTTP but not dATP or dCTP (Fig. 3 and data not shown), and both are sensitive to RNase A.

Immunoprecipitates of extracts from untagged strains often showed weak activity on oligonucleotide TELPT (see, for example, Figs. 3 and 6). Immunoprecipitates from yeast extracts usually contain significant amounts of nonspecific material, so some nonspecifically associated activity is not unexpected. We asked whether the nonspecifically precipitated activity was dependent on the presence of an *EST1* gene. A strain bearing an *est1*::*URA3* disruption was tested, and small

Table 1. Substrate oligonucleotides

Oligonucleotide	Sequence	Telomerase activity
TELPT (28 nt)	GTGTGTGGGTGTGTGTGTGGGTGTGTGT	+++
TELPG (27 nt)	GTGTGTGGGTGTGTGTGTGGGTGTGTG	+++
TELPGGG (28 nt)	TGTGTGTGTGTGTGGGTGTGTGTGTGGG	+++
HTELS3 (18 nt)	TTAGGGTTAGGGTTAGGG	−
HTELS4 (24 nt)	GGGTTAGGGTTAGGGTTAGGGTTA	−
MORIN (25 nt)	GCTTGCCAATCCGTCGAGCAGAGTT	+
HTELREP1 (26 nt)	CACACCCACACACCACACCCACACAC	−

TELPT, TELPG, and TELPGGG mimic the yeast telomeric G-rich strand, while HTELS3 and HTELS4 mimic the human telomeric G-rich strand. MORIN is a nontelomeric oligonucleotide which nevertheless serves as a good substrate for human telomerase (19). HTELREP1 mimics the yeast telomeric C-rich strand. +++ indicates robust addition of 10 or more nucleotides to the substrate oligonucleotide in telomerase reactions. + indicates the addition of a few nucleotides.

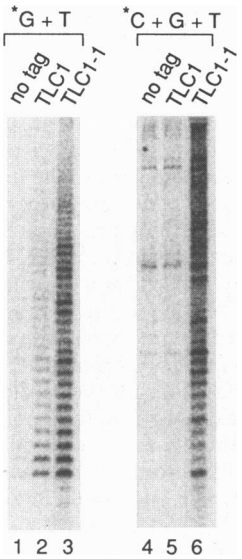

FIG. 4. The *TLC1-1* mutation allows incorporation of [α-^{32}P]dCTP. Immunoprecipitates were prepared from an untagged wild-type strain (lanes 1 and 4), from an Est1-tagged strain with wild-type *TLC1* (lanes 2 and 5), and from an Est1-tagged strain with mutant *TLC1-1* (lanes 3 and 6). Telomerase assays were carried out with TELPT as a substrate oligo, either in the presence of [α-^{32}P]dGTP plus unlabeled dTTP (lanes 1, 2, and 3) or in the presence of [α-^{32}P]dCTP plus unlabeled dGTP and dTTP (lanes 4, 5, and 6).

amounts of telomerase-like, RNase A-sensitive activity were found in immunoprecipitates (Fig. 6). The activity was quantitatively comparable with the trace activity in immunoprecipitates from an untagged *EST1* strain. Thus, this activity does not depend on Est1.

Little or no nonspecifically precipitated activity was seen when oligonucleotide TELPGGG was used as substrate (Fig. 6). Again, this argues that there are two different telomerase-like activities. Because the TELPGGG assay cannot be done in the absence of tagged Est1, we cannot tell whether this TELPGGG activity is present in an *est1* mutant.

DISCUSSION

Cells lacking *EST1* have similar or identical phenotypes to cells lacking *TLC1* (7, 11). Both kinds of mutants exhibit shortening

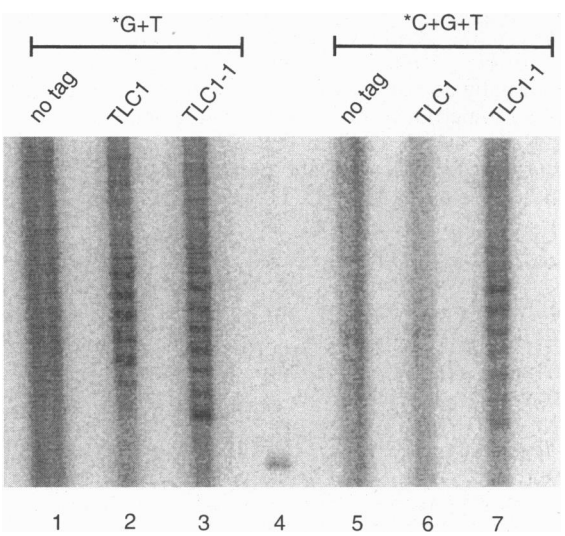

FIG. 5. *TLC1-1* allows incorporation of [α-^{32}P]dCTP. Lanes 1, 2, and 3 are as in Fig. 4, but with TELPGGG as substrate. Lane 4 is end-labeled TELPGGG. Lanes 5, 6, and 7 are as lanes 4, 5, and 6 in Fig. 4, but with TELPGGG as substrate.

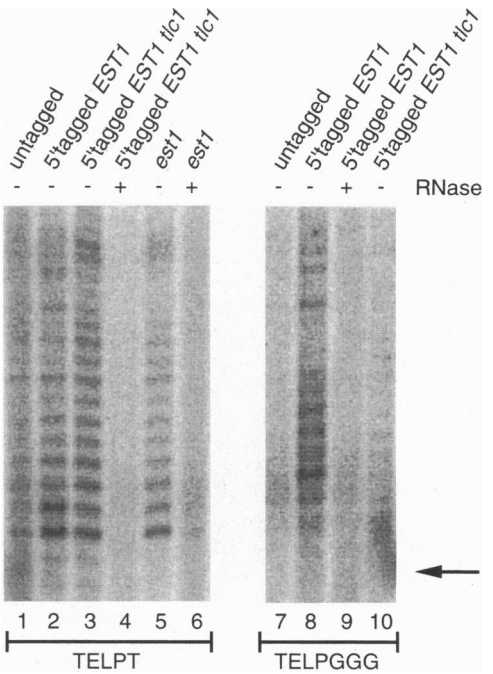

FIG. 6. Telomerase-like activities in *tlc1* and *est1* strains. Telomerase assays were done with [^{32}P]dGTP and unlabeled TTP by using immunoprecipitates from the indicated strains and oligonucleotide TELPT (lanes 1–6) or TELPGGG (lanes 7–10). RNase A (10 ng) was added to the reaction mixtures shown in lanes 4, 6, and 9. The arrow indicates the position of end-labeled TELPGGG.

telomeres, and both senesce—i.e., have a limited replicative capacity. This is consistent with the idea that Est1 is important for telomerase function *in vivo*. We now show that Est1 is physically associated with Tlc1 and with a telomerase-like activity. Evidence that the activity is telomerase includes the nucleotide specificity, the primer specificity, and the RNase A sensitivity, but the strongest evidence is that when the template region of *TLC1* is changed to include G residues, the activity will begin to incorporate dCTP residues.

Curiously, we seem to have found two activities. One acts on the TELPGGG oligonucleotide (and probably others), while the second acts on TELPT but not on TELPGGG. The TELPGGG activity is absent in a *tlc1* strain and is not nonspecifically immunoprecipitated in an untagged *EST1* strain. In contrast, TELPT activity is present even in a *tlc1* null strain, and some is nonspecifically immunoprecipitated from an *est1* extract or an untagged *EST1* extract. We are confident that the activity seen on oligonucleotide TELPGGG is a yeast telomerase because it is absent in a *tlc1* null strain, and it incorporates dCTP residues in the *TLC1-1* mutant. However, we are uncertain of the nature of the TELPT activity seen in a *tlc1* strain. It is telomerase-like in that it is sensitive to RNase A, and it has the correct nucleotide and substrate specificity, but perhaps other kinds of activities could also have these characteristics. If this activity is indeed a telomerase, then the fact that it persists in a *tlc1* deletion strain suggests that there may be a second telomerase RNA. Until a second such RNA is found and appropriate mutations made and tested, we will not be sure whether the *TLC1*-independent activity seen with oligonucleotide TELPT is a telomerase or some other kind of activity.

We note that neither *TLC1* nor *EST1* is an essential gene in the usual sense; rather, mutants "senesce" over many generations. This has been explained by saying that telomeric sequences are lost only very gradually from the end of the chromosome, presumably because of the end-replication problem (20, 21). The loss is surprisingly slow: Lundblad and

Szostak (11) estimated that an *est1* strain lost about 100 bp of telomeric sequence per 25 generations. This rate of 4 bp per generation implies that RNA primers of 8 bp or less are sufficient for DNA replication. Furthermore, "survivors" occur in *est1* populations, which are due to amplification of subtelomeric repeat sequences, and these can be grown indefinitely (12). These survivors apparently still have some TG_{1-3} at their chromosome ends even after hundreds of generations in the absence of *est1* (12). It is possible that the slow senescence in *est1* and in *tlc1* mutants and the continued presence of TG_{1-3} repeats after many generations of growth in *est1* survivors is due to the continued presence of some kind of telomerase activity. A strain completely lacking telomerase might die more rapidly.

Our finding of two telomerase-like activities may help explain other recent results. Yeast telomerase activity has recently been detected by other investigators (22–24). In one study, an oligonucleotide ending in GGG was used as substrate, and activity was absent when *tlc1* strains were used (22). In a second study, an oligonucleotide ending in GTG was used, and some telomerase-like activity was present in *tlc1* null strains (23). Our results with oligonucleotides TELPGGG and TELPT suggest that these apparently conflicting results may be due to the different oligonucleotides used as substrates. These two studies also differed in that one found activity in *est1* strains (22), while the other did not (23). We find that the *TLC1*-independent activity is present in *est1* strains. However, while the *TLC1*-dependent activity is clearly associated with Est1, we have no information on whether it is dependent on *EST1* because it is not nonspecifically precipitated, and so we cannot assay for it in an *est1* mutant.

Five percent or less of the Tlc1 RNA could be coimmunoprecipitated with Est1. Although there are other interpretations, this may mean that only a fraction of the cell's telomerase is associated with Est1. Est1 may not be a catalytic subunit, but rather an accessory protein, perhaps responsible for targeting the complex to telomeres or for regulation of the complex.

The recent identification of the yeast telomerase RNA (7), and now the identification of yeast telomerase activity and an associated protein (refs. 22–24 and this work), will allow rapid advances in the study of this enzyme by using the molecular and genetic tools available in yeast.

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