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Species-specific and sequence-specific recognition of the dG-rich strand of telomeres by yeast telomerase

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ABSTRACT

A gel mobility shift assay was developed to examine recognition of yeast telomeres by telomerase. An RNase-sensitive G-rich strand-specific binding activity can be detected in partially purified yeast telomerase fractions. The binding activity was attributed to telomerase, because it co-purifies with TLC1 RNA and telomerase activity over three different chromatographic steps and because the complex co-migrates with TLC1 RNA when subjected to electrophoresis through native gels. Analysis of the binding specificity of yeast telomerase indicates that it recognizes the G-rich strand of yeast telomeres with high affinity and specificity. The K_d for the interaction is ~3 nM. Single-stranded G-rich telomeres from other species, such as human and *Tetrahymena*, though capable of being extended by yeast telomerase in polymerization assays at high concentrations, bind the enzyme with at least 100-fold lower affinities. The ability of a sequence to be bound tightly by yeast telomerase *in vitro* correlates with its ability to seed telomere formation *in vivo*. The implications of these findings for regulation of telomerase activity are discussed.

INTRODUCTION

Telomerase is a ribonucleoprotein (RNP) that is responsible for the synthesis of the dG-rich strand of telomere terminal repeats (1–3). The enzyme was initially identified in *Tetrahymena thermophila*, using an assay that exploited the ability of telomerase to extend telomere-like oligodeoxynucleotides in the presence of dGTP and dTTP (4). Telomerase was subsequently found to be an unusual reverse transcriptase containing an integral RNA component, a small segment of which acts as the template for synthesis of the dGT-rich strand of telomeric repeats (5). Some telomerase can extend primers in a processive fashion, adding many copies of the telomeric repeats before dissociating (6,7). Telomerase can also cleave the input primer under certain conditions (8–10), a property that is shared by a number of DNA and RNA polymerases.

Telomerase activity has been detected in a wide range of organisms, including protozoa (3), yeast (8,11–13), mouse (14), *Xenopus* (15) and human (7). Genes encoding the RNA component of the enzyme complex have been cloned for many telomerases, such as that of yeast and human (16,17). Recently some of the polypeptide components of telomerase were cloned. In particular, a yeast protein known as Est2p and homologs of Est2p in *Euplotes aediculatus*, human and *Schizosaccharomyces pombe* were shown to be the catalytic components of the respective telomerases (18–21); these polypeptides exhibit significant homology to other reverse transcriptases and mutations that alter Est2p residues that are conserved among reverse transcriptases abolish telomerase activity *in vitro* and telomerase function *in vivo*. In addition, two polypeptides, p80 and p95, that co-purify with *Tetrahymena* telomerase have been cloned and been shown to interact with telomerase RNA and the DNA primer respectively (22). Mouse and human homologs of p80 have also been identified and been shown to associate with the respective telomerases (23,24). These recent developments should greatly facilitate structure–function analysis of telomerase.

How telomerase recognizes the ends of chromosomes *in vivo* is not clearly understood. *In vitro* at high primer concentrations telomerase appears capable of extending promiscuously all oligonucleotides that have a high percentage of dG residues. As few as 3 nucleotides at the 3′-end of the primer need to form a hybrid with telomerase RNA to allow polymerization to proceed (25). In the case of ciliated protozoa even AT-rich non-telomeric oligonucleotides can support primer extension efficiently (26). These findings have led to the suggestion that telomerase does not bind telomeres with high sequence specificity. However, only in the case of *Tetrahymena* was binding of telomerase to primers examined in a direct assay and the results appear consistent with a loose sequence preference for telomerase (27).

To test the generality of promiscuous binding by telomerase we developed a gel mobility shift assay for the enzyme from *Saccharomyces cerevisiae*. Although yeast telomerase was not purified, an RNA-dependent complex that contained the yeast telomerase RNA (TLC1 RNA) could be readily identified using active fractions and was attributed to telomerase–primer interaction. Surprisingly, analysis of the binding specificity of yeast telomerase indicates that it recognizes the dG-rich strand of yeast telomeres with high affinity and specificity. In addition, high affinity binding to a primer does not correlate with the primer's ability to support extension. However, high affinity binding appears to correlate with the primer's ability to seed telomere formation

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Table 1. Oligonucleotides

Oligo	Sequence
TEL15	TGTGTGGTGTGTGGG
TEL19*	TGTGTGACTGTGTGGGTGT
TEL24	TGTGTGGGTGTGTGGGTGTGTGGG
(TG)n	TGTGTGTGTGTGTGTG
(TGG)n	TGGTGGTGGTGGTGGG
(TGGG)n	TGGGTGGGTGGGTGGG
(TAG_{1-3})n	TAGGGTAGTAGTAGGG
PTEL16	GTTACAGGTTACAGGG
TETRA1	GGGTTGGGGTTGGGGTTGGGGTTG
TETRA2	TTGGGGTTGGGGTTGGG
OXYT1	GTTTTGGGGTTTTGGG
ARAB1	GTTTAGGGTTTAGGG
HS1	TTAGGGTTAGGGTTAGGGTTAGGG
HS2	GGGTTAGGGTTAGGG

in vivo. The implications of these results for telomerase function and regulation of telomerase activity are discussed.

MATERIALS AND METHODS

Strain and oligonucleotides

The haploid *S.cerevisiae* strain DG338 (a gift of D.Garfinkel, National Cancer Institute) was used for derivation of active telomerase. The oligonucleotides used are listed in Table 1.

Purification of *S.cerevisiae* telomerase

Derivation of whole cell extracts and the active DEAE fractions was as described elsewhere (9,28). The telomerase activity was further purified on a heparin column, a blue column, a Q column and a Phenyl-Sepharose column as follows. Telomerase-containing DEAE fractions were pooled, diluted with TMG-10(0) (28) to a conductivity of 30 mSi, loaded onto an Affi-Gel Heparin Gel (BioRad) column and the activity eluted with 2 column vol TMG-10(700). Telomerase-containing fractions were pooled and loaded directly onto an Affi-Gel Blue Gel (BioRad) column. The column was washed successively with 2 column vol each TMG-10(500) and TMG-10(1000) and the activity eluted with 2 column vol TMG-10(1000), 1% Triton X-100. Telomerase-containing fractions were pooled, diluted with TMG-10(0) to a conductivity of 40 mSi, loaded onto a Bio-Scale Q (BioRad) column and the activity eluted with 10 column vol of a gradient from TMG-10(400) to TMG-10(1500). Telomerase-containing fractions (conductivity ~110 mSi) were pooled and loaded directly onto a Phenyl-Sepharose (Pharmacia) column. The column was washed successively with 2 column vol each of TMG-10(500) and TMG-10(0) and the activity eluted with 2 column vol TMG-10(0), 1% triton X-100.

For the glycerol gradient fractionation active telomerase from the DEAE column was concentrated 10-fold by use of Centricon-30 (Amicon) and applied to a 20–50% glycerol gradient in TMG-10(200). The gradient was spun at 35 000 r.p.m. at 4°C for 20 h and 15 equal volume fractions collected. The positions of Blue Dextran 2000 and ribosomal particles run in a parallel gradient were used as molecular size standards.

Primer extension assay

Primer extension assays were carried out using 5 µM primer oligodeoxynucleotides as described elsewhere (28). For quantification of activity the signals from all labeled and RNase-sensitive products (including those that are shorter than the input primer) are summed.

Gel mobility shift assay

DNA primers used for gel mobility shift experiments were radiolabeled with T4 polynucleotide kinase and purified over a Nick Column (Pharmacia). Complex formation between yeast telomerase and DNA primer was monitored using a previously described gel mobility shift protocol (29). Briefly, partially purified telomerase was incubated with 5 nM (0.4 ng) labeled primer in 16 µl buffer containing 10 mM Tris–HCl, pH 8.0, 2 mM $MgCl_2$, 12% glycerol and 50 ng poly(dI·dC). Where indicated, cold competitor oligonucleotides were added before addition of telomerase. The binding was carried out at 4°C for 20 min and complex formation monitored by electrophoresis through a 4% polyacrylamide gel with running buffer containing 25 mM Tris–HCl, pH 8.3, 190 mM glycine, 5 mM $MgCl_2$ and 1 mM EDTA. The gel was cast in running buffer supplemented with 10% (v/v) glycerol and 0.5 mM DTT.

RNA analysis

To follow migration of TLC1 RNA in native gels the polyacrylamide gel used in the mobility shift assay was supplemented with 0.5% agarose and used for separation of the complexes. Following electrophoresis the part of the gel to be analyzed for localization of TLC1 RNA was rinsed briefly in 75 mM Tris–glycine and soaked in 50% urea, 25 mM Tris–glycine, 0.5 mM EDTA with gentle shaking for 30 min. The nucleic acids within the gel were transferred to Hybond-N membrane in 6.0 mM trisodium citrate, 8 mM sodium phosphate (dibasic) at 250 mA for 16 h at 4°C (30). The blot was then probed with a labeled TLC1 DNA fragment using standard protocols.

RESULTS

Identification of a yeast telomerase–telomere complex

For derivation of active yeast telomerase we fractionated crude extracts over a DEAE-agarose column, as described by Cohn and Blackburn (9). A high salt eluate from this column has a robust RNase-sensitive polymerization activity that is dependent upon the RNA component of yeast telomerase, TLC1 (9,28). Preparation of the fraction from a strain harboring point mutations in the TLC1 template region yielded an activity that has the expected property for nucleotide utilization; substitution of a GG dinucleotide for an AC dinucleotide in the RNA template resulted in a fraction that incorporates dCMP in addition to dGMP and dTMP (28). Because this fraction appears largely devoid of other contaminating activities, we have used it for further purification and for subsequent biochemical assays.

To examine binding of yeast telomerase to single-stranded telomeres in the absence of polymerization we sought to develop a gel mobility shift assay. As shown in Figure 1A, when a

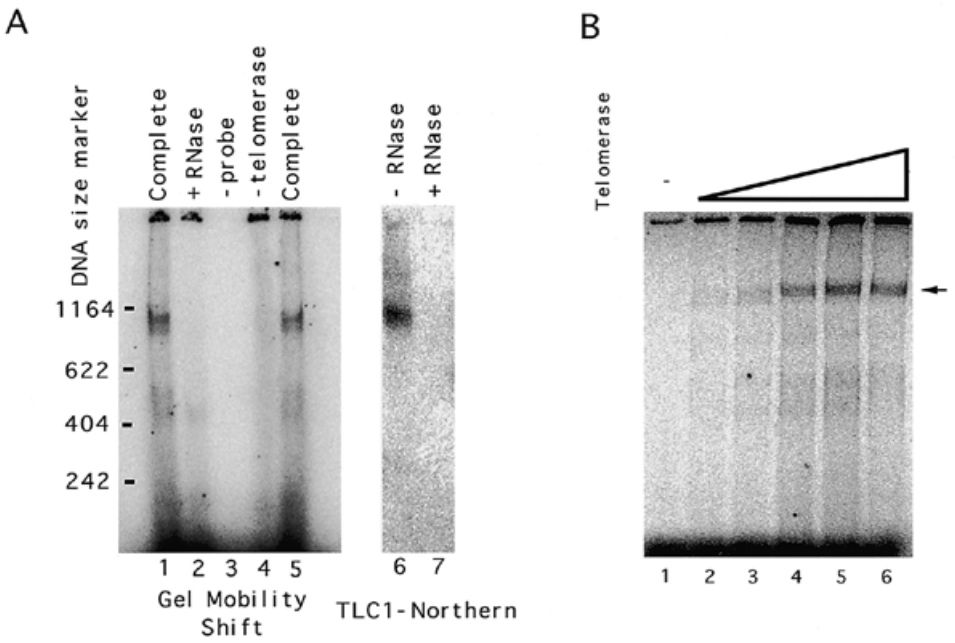

Figure 1. Yeast telomerase–telomere interaction can be detected by a gel mobility shift assay. (**A**) (Left) Gel mobility shift assays were carried out using labeled TELI5 (TGTGTGGTGTGTGGG) as probe and yeast telomerase that has been purified on a DEAE column. To validate the specificity of the observed complex the telomerase fraction was pre-incubated with 10 ng RNase A at 20°C for 10 min prior to binding (+RNase), the probe was omitted (–probe) or the telomerase fraction was omitted (–telomerase). (Right) Telomerase fractions that have been incubated in the absence (–RNase) or presence (+RNase) of RNase A were electrophoresed into the same native gel as that used for the mobility shift assays. Following electrophoresis the telomerase complex was denatured *in situ*, transferred to nylon membrane and probed with labeled TLC1 DNA. (**B**) Increasing amounts of telomerase (0, 0.005, 0.01, 0.02, 0.04 and 0.06 nM as estimated by the concentration of TLC1 RNA) were mixed with 2 nM labeled TEL24 at room temperature for 10 min prior to gel mobility shift analysis.

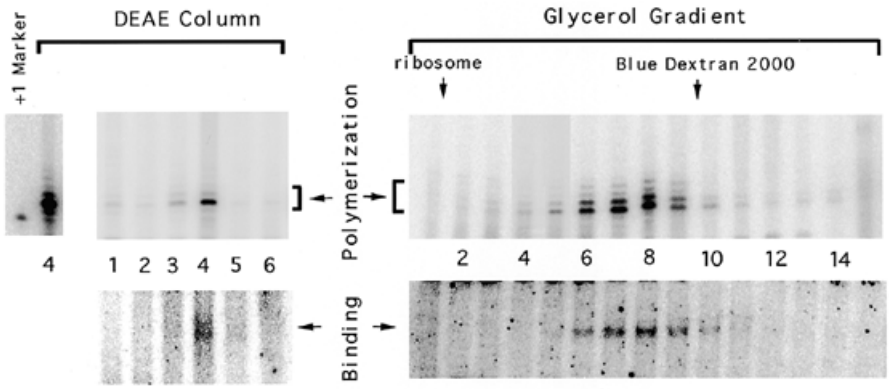

Figure 2. Co-purification of the primer extension and binding activity of yeast telomerase. Fractions from a DEAE column (**A**) or a glycerol gradient (**B**) were subjected to both polymerization and binding assays. Polymerization assays were done using oligo TEL15(–12)G→C (TGTCTGGTGTGTGGG) as primer oligonucleotide and the results are shown in the top panel. Telomerase signal is indicated by brackets to the left or right of the panel. A separate polymerization assay using DEAE fraction 4 was run alongside a 'primer + 1' marker to give a rough estimate of the lengths of the products. The 'primer + 1' marker was synthesized by labeling TEL15 with terminal transferase and radioactive cordycepin. Binding assays were done using labeled TEL15 (TGTGTGGTGTGTGGG) as probe and the results shown in the bottom panel. The position of the RNase-sensitive complex in the binding assays is indicated by an arrow to the left or right of the panel. Fraction numbers are shown between the polymerization and binding panels and the positions of molecular weight standards for the glycerol gradient are marked at the top of the upper panel.

telomerase-containing fraction was incubated with the dG-rich strand of yeast telomeres and then subjected to electrophoresis through a 4% magnesium-containing gel a major low mobility complex can be visualized (lanes 1 and 5). Consistent with the complex being due to telomerase RNP, formation of the complex can be prevented by pre-incubating the fraction with RNase A (lane 2) or proteinase K (data not shown). Pre-incubating the fraction with RNase A in the presence of an RNase inhibitor resulted in retention of the complex (data not shown). Besides the dual sensitivity to degrading enzymes, several additional lines of evidence support the complex being due to telomerase. First, when telomerase RNP alone was subjected to electrophoresis through the same gel its mobility as determined by the location of TLC1 RNA was identical with that of the probe-containing complex, indicating that the probe was likely shifted by binding to telomerase (Fig. 1A, compare lanes 1 and 5 with 6). (The presence of a 15 nt DNA probe in lane 1 was not expected to alter the mobility of the telomerase RNP that contains a 1300 nt RNA.) Second, the complex forming

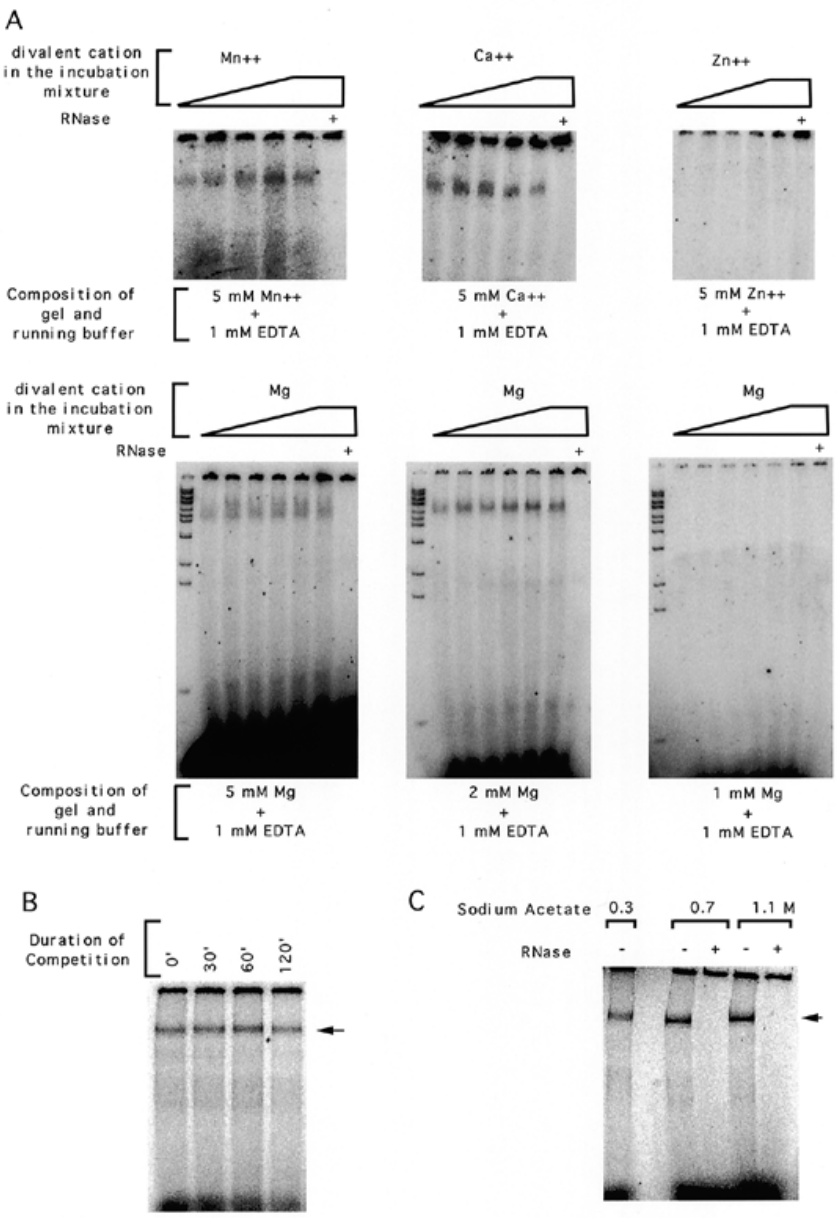

Figure 3. Divalent metal requirement for and salt resistance of the telomerase–telomere complex. (**A**) The gel mobility shift assays were carried out using labeled TEL15 as probe under a variety of conditions. The divalent cations used for casting the gels are indicated at the bottom of each panel and the divalent cations used in the binding reaction indicated at the top of each panel. The cation concentrations in the binding reactions for all of the top panels were 0.3, 1.0, 1.6, 2.5, 4.8 and 4.8 mM (left to right). Those for all of the bottom panels were 0.3, 0.8, 1.3, 2.3, 3.3, 5.3 and 5.3 mM (left to right). One reaction in each panel was pretreated with RNase (+) before binding and electrophoresis. (**B**) Binding reactions were carried out using 5 nM labeled TEL24 and 0.06 nM telomerase. After incubation at room temperature for 10 min a 2000-fold molar excess of unlabeled TEL24 was added and incubation continued for the indicated time prior to native gel electrophoresis. (**C**) Binding reactions were carried out using 5 nM labeled TEL24 and 0.06 nM telomerase in the presence of different sodium acetate concentrations as indicated at the top of the panel. In some assays the telomerase fraction was pretreated with RNase A prior to binding (+RNase). After incubation at room temperature for 10 min a 2000-fold molar excess of unlabeled TEL24 was added to each mixture prior to native gel electrophoresis. RNase-sensitive complexes were indicated by an arrow to the right of the panel.

activity co-purified with polymerization activity over three different purification steps: DEAE column, heparin column and glycerol gradient sedimentation (Fig. 2 and data not shown). Third, no complex was observed when the C-rich strand of yeast telomere or duplex yeast telomere was used as probe, consistent with telomerase recognizing and extending only the G-rich strand of telomeres (28; data not shown). The affinity of the telomerase RNP for single-stranded yeast telomeres was estimated by a titration experiment (Fig. 1B). If one assumes that all TLC1 RNA in the fraction (as quantified by dot blotting assays; data not shown) was assembled into active RNP, then the the affinity of telomerase for a canonical yeast telomere (TEL24 in Table 1) is estimated to be ~3 nM.

Requirements for formation and characteristics of the telomerase–telomere complex

To gain insights into the mechanisms of complex formation we investigated the binding reaction as a function of divalent metal ion

and salt concentrations. Stable binding of yeast telomerase to the telomere required moderate concentrations of magnesium in the gel. As shown in Figure 3A, when the gel and running buffer contained 2–5 mM magnesium and 1 mM EDTA an RNase-sensitive complex can be visualized (bottom three panels). However, reducing the magnesium concentration in the gel to 1 mM while retaining 1 mM EDTA resulted in disappearance of the complex. The magnesium requirement can be satisfied by manganese and calcium, but not by zinc (Fig. 3A, top three panels). When EDTA was omitted from the gel and running buffer, a lower concentration of magnesium (1 mM) was sufficient for complex formation (data not shown). In contrast to the divalent cation requirement in the gel, complex formation was unaffected by the concentration of divalent cations in the initial incubation mixture (as indicated at the top of each panel). Interestingly, the complex visualized in the presence of 5 mM magnesium is considerably more heterogeneous than that in 2 mM magnesium, suggesting that the complex may adopt a greater number of conformations at higher magnesium concentrations.

The telomerase–telomere complex is exceptionally stable, with a half-life >2 h. As shown in Figure 3B, when a 2000-fold molar excess of unlabeled TEL24 is added to the incubation mixture following binding of telomerase to labeled TEL24 ~80% of the signal can still be observed after a further 2 h incubation. This slow dissociation allowed us to analyze formation of the complex in high salt. In the reactions shown in Figure 3C telomerase fractions were first mixed with labeled TEL24 in the presence of high concentrations of sodium acetate for 10 min. A 2000-fold molar excess of unlabeled TEL24 was then added to prevent further binding and the resulting mixture subjected to gel mobility shift analysis. Even if the excess sodium acetate were to diffuse quickly under electrophoretic conditions, no additional binding of telomerase to labeled TEL24 would be expected because of the presence of excess competitor. Consequently, our ability to visualize RNase-sensitive complexes in these reactions indicates that binding of telomerase to telomeres can occur even in the presence of up to 1.1 M sodium acetate (Fig. 3C). This in turn suggests that there may be significant hydrophobic interactions within the complex or that the telomerase RNA contributes significantly to telomere binding. (Formation of nucleic acid complexes are facilitated by charge neutralization.)

Species-specific recognition of telomeres by yeast telomerase

We tested the sequence specificity of binding by yeast telomerase using a variety of dG-rich oligonucleotides (Table 1). Single-stranded oligonucleotides containing telomere repeats from *S.pombe* (PTEL16), *Tetrahymena* (TETRA1 and TETRA2), *Oxytrica* (OXYT1), *Arabidopsis* (ARAB1) and human (HS1 and HS2) were used as competitors in the gel mobility shift assay and the affinity of these oligonucleotides relative to a yeast oligonucleotide (TEL15) estimated by the amount required for half-maximal competition. As shown in Figure 4 (top panels) and Table 2, none of the telomeres derived from other species competed significantly for binding, even when present at 100-fold molar excess. Since *S.cerevisiae* telomeres contain a number of degenerate repeats (e.g. TG, TGG or TGGG), we also tested these different repeats in the competition assay. As shown in Figure 4 (bottom panel) and Table 2, the dinucleotide repeat $(TG)_n$ competed well for telomerase binding. In contrast, primers made up of the trinucleotide

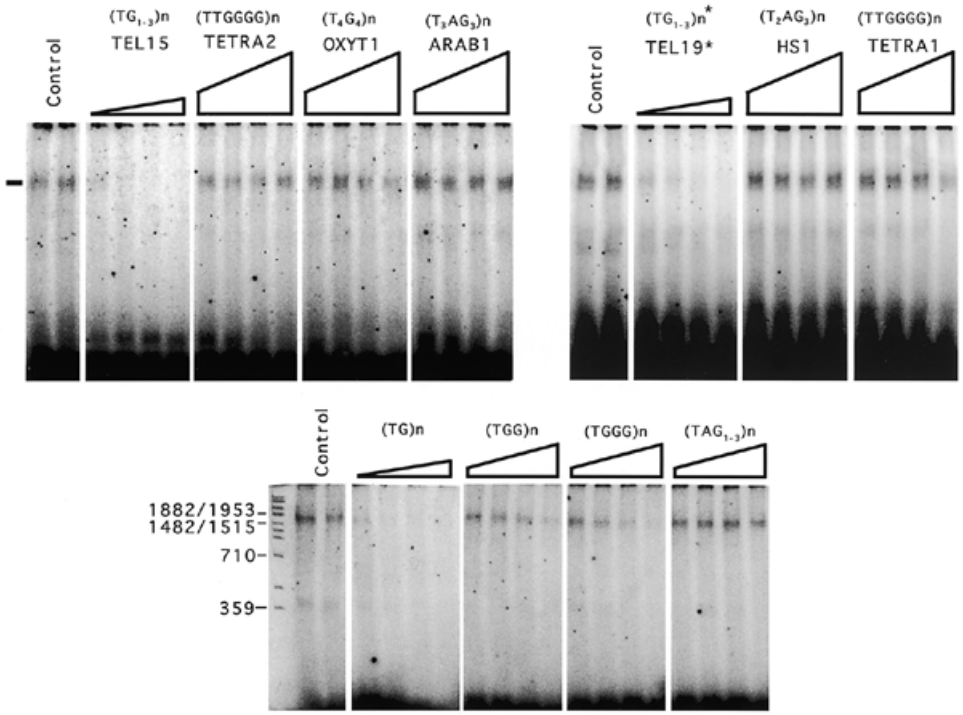

Figure 4. Yeast telomerase selectively recognizes yeast telomeres. Competition gel mobility shift assays were carried out using labeled TEL15 as probe and partially purified yeast telomerase. Increasing amounts of various unlabeled oligonucleotides were used as competitors as indicated at the top of each panel. The nature of the repeat, if not clear from the name of the oligonucleotide, was also shown at the top. The molar excesses of competitor to probe used were: for TEL15 and TEL19*, 5-, 15-, 45- and 135-fold (left to right); for $(TGG)_n$, $(TGGG)_n$ and $(TAG_{1–3})_n$, 25-, 75-, 225- and 675-fold (left to right); for TETRA2, OXYT1, ARAB1, HS1, TETRA1, 100-, 300-, 900- and 2700-fold (left to right). Double-stranded DNA size standards were run alongside the mobility shift assays in the bottom panel.

Table 2. Relative binding and polymerization activites

Oligo	Molar ratio required for 1/2 competition#	Relative polymerization*
TEL15	1.0 : 1	1
TEL19*	2.3 : 1	N.D.±
TEL24	1.0 : 1	N.D.
(TG)n	1.0 : 1	1.2
(TGG)n	350 : 1	12
(TGGG)n	230 : 1	5
$(TAG_{1-3})n$	1300 : 1	48
PTEL16	> 2500 : 1	11
TETRA1	~ 1700 : 1	N.D.
TETRA2	> 2500 : 1	23
OXYT1	~ 2500 : 1	26
ARAB1	> 2500 : 1	19
HS1	> 1700 : 1	11
HS2	N.D.	22

#Molar ratio of unlabeled oligonucleotides to labeled TEL15 required for 50% competition of the signal observed with labeled TEL15 alone in the gel mobility shift assay.

*With each primer the total radioactivity incorporated into all of the labeled products was determined using a PhoshphorImager. The results are normalized against the signal observed with TEL15.

±Not determined.

repeat d(TGG) $[(TGG)_n]$, the tetranucleotide repeat d(TGGG) $[(TGGG)_n]$ and a repeat with dA insertions $[(TAG_{1-3})_n]$ had much lower affinities for telomerase. We conclude that yeast telomerase exhibits stringent sequence specificity for its own irregular telomeric repeats and for the dinucleotide repeat $(TG)_n$. This finding is supported by another study showing that selected point mutations in a canonical yeast telomere oligonucleotide can also drastically reduce its binding affinity for telomerase (28).

Lack of correlation between binding and extension by yeast telomerase

To determine if the binding specificity of yeast telomerase correlates with polymerization specificity we subjected the same set of oligonucleotides to the standard primer extension assay. Unexpectedly, non-yeast primers were found to support greater polymerization by yeast telomerase in general. For example, under the standard reaction conditions the human HS1 primer was 11 times better and the *Oxytrica* OXYT1 primer 26 times better than the yeast primer TEL15 (Fig. 5A and Table 2). Greater polymerization was also observed for oligonucleotides corresponding to two sub-elements of the yeast telomeric repeats $[(TGG)_n$ and $(TGGG)_n$; Table 2]. To confirm that the signals observed were entirely due to telomerase we analyzed incorporation of additional nucleotides by the polymerization activity. As expected, little dCMP incorporation was observed with a non-yeast primer (OXYT1) when telomerase, derived from a TLC1 strain was used. Mutating two of the template residues of TLC1 RNA from

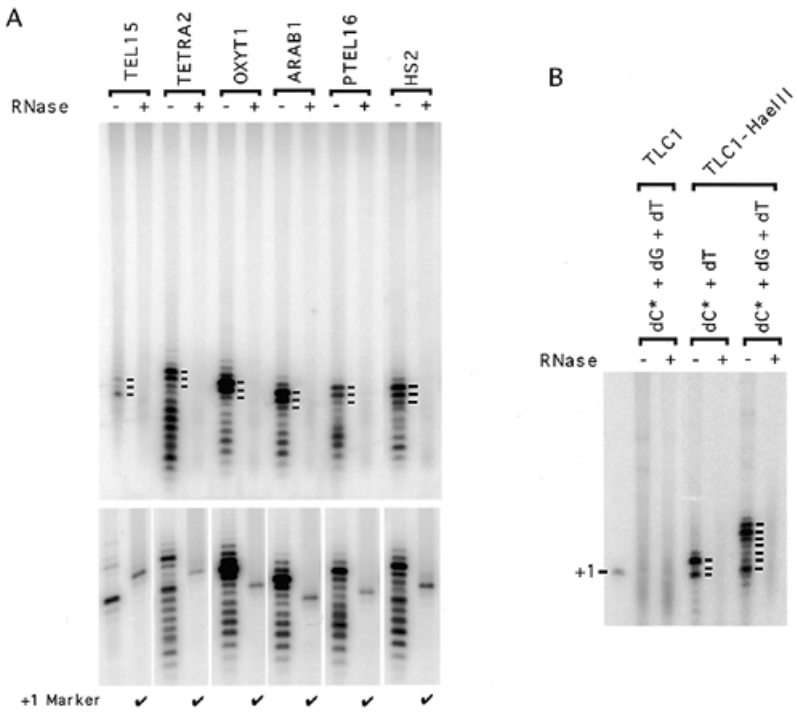

Figure 5. Non-yeast primers can be efficiently extended by yeast telomerase. (**A**) Primer extension assays were carried out using DEAE fractions and a number of different primers as indicated at the top of the panel. As controls fractions were pretreated with RNase A (+) before addition of primer and nucleotides. The 'primer + 1' to 'primer + 3' products are indicated by horizontal lines to the right of the lanes. In a separate series of assays shown at the bottom, the reaction products were run alongside 'primer +1' markers for size determination. The 'primer + 1' markers were synthesized by labeling each primer with terminal transferase and radioactive dTTP. (**B**) Primer extension assays were carried out using OXYT1 as primer and DEAE fractions derived from either wild-type (TLC1) or mutant (TLC1-*Hae*III) strains. The mutant TLC1 RNA is expected to support synthesis of CCTGGTG assuming optimal alignment. The combinations of labeled (*) and unlabeled nucleotides used for the reactions are indicated at the top of the panel. A 'primer + 1' marker, synthesized by labeling OXYT1 with terminal transferase and radioactive cordycepin, is shown to the left of the reaction products.

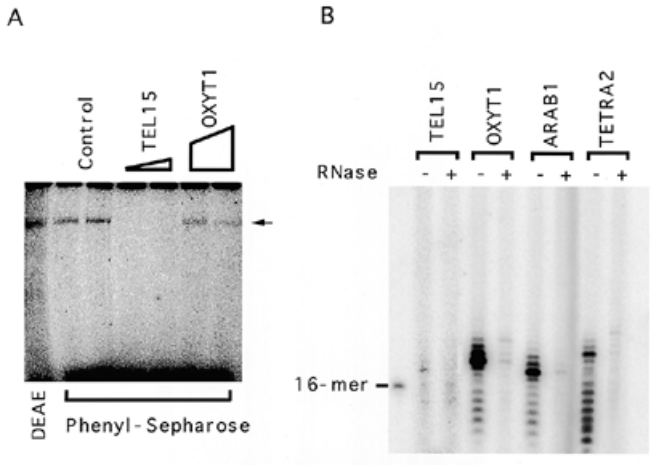

Figure 6. Extensively purified telomerase exhibits similar sequence specificity for binding and polymerization as partially purified enzyme. (**A**) Gel mobility shift assays were carried out using labeled TEL15 as probe and either DEAE or Phenyl-Sepharose fractions. Unlabeled TEL15 (5- and 15-fold molar excess) or OXYT1 (900- and 2700-fold molar excess) was included in some reactions as competitors as indicated at the top of the figure. (**B**) Primer extension assays were carried out using Phenyl-Sepharose fractions and various oligonucleotides as primers. As controls fractions were pretreated with RNase A (+) before addition of primer and nucleotides. A 16 nt size marker, synthesized by labeling TEL15 with terminal transferase and radioactive cordycepin, is shown in the leftmost lane.

r(CA) to r(GG), however, led to incorporation of dCMP, as expected (TLC1-*Hae*III fraction; Fig. 5B). This result indicates that the DNA synthesis supported by the non-yeast primers is unlikely to be due to a contaminating DNA polymerase. Thus for the yeast enzyme the primer binding specificity does not match primer elongation specificity, at least *in vitro*.

Sequence-specific recognition is the property of a tightly associated subunit(s) of yeast telomerase

Because the bulk of binding and elongation studies have been done using partially purified yeast telomerase, we questioned whether the presence of auxiliary factors in the fraction may have altered the properties of yeast telomerase. Perhaps the properties of yeast telomerase can be attributed to some loosely bound or entirely extrinsic accessory factors. To test this hypothesis we further purified yeast telomerase on four columns and subjected the resulting fraction to binding and elongation analysis. As shown in Figure 6, extensively purified yeast telomerase (Phenyl-Sepharose fraction) continued to exhibit high sequence specificity for binding and a loose sequence requirement for polymerization. For example, primers with the telomeric repeat from *Oxytrica* (OXYT1) continued to be bound poorly by yeast telomerase (Fig. 6A). Non-yeast primers, however, continued to serve as good substrates for elongation by telomerase (Fig. 6B). We conclude that the binding and polymerization properties of yeast telomerase as determined using partially purified fractions are likely due to a tightly associated subunit or subunits.

DISCUSSION

We have shown that partially purified yeast telomerase RNP recognizes the dG-rich strand of the yeast telomere terminal repeat with high affinity and specificity. Although the enzyme preparation used for binding assays was not homogeneous, sequence-specific recognition is likely due to a tightly associated subunit or subunits rather than some loosely associated auxiliary factor. First, even extensively purified telomerase exhibits similar sequence specificity. Second, complex formation can be observed in 1.1 M sodium acetate, a salt concentration that is often sufficient to dissociate loosely bound factors. Finally, the mobility of the complex is similar to that of 1 kb double-stranded DNA (Fig. 1A) and only slightly lower than naked TLC1 RNA (data not shown), suggesting that the complex does not contain many polypeptides in addition to the essential ones.

The subunits of yeast telomerase responsible for high affinity and sequence-specific binding are not known. Because formation of the particular complex is sensitive to both RNase and proteinase K pretreatment, high affinity binding probably involves both an RNA and a protein component(s). Earlier crosslinking studies on the *Euplotes* telomerase suggest that both the protein catalytic subunit (Est2p homolog) and telomerase RNA come into close contact with the upstream region of the DNA primer, consistent with participation of both in primer binding (31). In addition, a putative yeast telomerase subunit known as Est1p has been shown to bind single-stranded yeast telomeres on its own, consistent with its participation in primer recognition by telomerase (32). However, the affinity of binding for Est1p alone is 250 nM, considerably higher than that of telomerase RNP. Furthermore, the sequence requirement for binding by Est1p appears to be less stringent than telomerase RNP. Thus if Est1p participates in recognition of telomeres by telomerase it must do so in concert with other components. Like Est1p, the *CDC13* gene product has also been shown to bind single-stranded yeast telomeres with high affinity (33,34). However, current evidence indicates that Cdc13p is likely to be a telomere binding protein rather than a telomerase component *in vivo*.

The divalent cation requirement for detection of a yeast telomerase–telomere complex suggests that these cations either participate directly in binding or are required to maintain the correct conformation of the RNP. Indeed, a number of large RNAs have been shown to require magnesium for proper folding (35). In contrast to the recognition properties of yeast telomerase, the *Tetrahymena* enzyme has been shown to bind a number of dG-rich oligonucleotides with distinct sequence repeats; in addition to d(TTGGGG)$_3$, which contains the canonical *Tetrahymena* telomere repeat, the *Tetrahymena* enzyme can also bind d(TTAGGG)$_3$, d(TG)$_9$ and d(TGTGTGGG)$_2$TG with high affinity (27). In addition, stable binding of *Tetrahymena* telomerase to primer oligonucleotides can be observed even in the absence of divalent cations and in the presence of 0.2 mM EDTA (27). Perhaps the ciliate telomerase RNAs, being smaller, do not need magnesium for proper folding. Alternatively, the molecular mechanisms of binding may be significantly different between the yeast and the ciliate telomerases. That ciliate telomerase should have evolved a different primer recognition mechanism is not at all surprising in the light of its role in the *de novo* synthesis of telomeres during the normal life cycle of the organism. In contrast, there is no need for yeast telomerase to extend non-homologous telomeric ends or non-telomeric ends for normal chromosome maintenance.

The ability of various GT-rich sequence repeats to serve as the seed for telomere formation in yeast has been carefully examined in two earlier studies (36,37). When these *in vivo* studies are compared with our *in vitro* assays a remarkable correlation can be discerned

between the ability of a sequence to be bound by telomerase and its ability to contribute to telomere healing. For example, the dinucleotide repeat oligo $(TG)_n$ is bound with high affinity by the enzyme and a comparable repeat directs high efficiency healing *in vivo*. In addition, the trinucleotide repeat oligo $(TGG)_n$, the tetranucleotide repeat oligo $(TGGG)_n$ and the irregular repeat oligo $(TAG_{1–3})_n$ are weakly bound by telomerase *in vitro* and comparable repeats are unable to promote telomere healing *in vivo* (Fig. 4, bottom panel and Table 2). In contrast to the binding results, the ability of a sequence to prime DNA synthesis *in vitro* does not correlate with the ability to direct telomere healing. All four primers tested in this series supported significant polymerization by yeast telomerase *in vitro* (Table 2). Thus the binding assay appears to more accurately reflect the relative activity of different sequences for telomerase extension *in vivo* than the standard polymerization assay. Perhaps *in vivo* the binding of telomeric ends by telomerase is a limiting step for extension. Similar phenomena may occur in humans, where seeding of telomeres *in vivo* has been shown to require homologous telomeric repeats (38). In addition to binding by telomerase, the ability of a sequence to be recognized by telomere binding proteins may also contribute to telomere healing (36–38). However, because the $(TG)_n$ oligo lacks a binding site for RAP1, the major yeast telomere binding protein yet can support telomere formation at high efficiency, the presence of a binding site for RAP1 is not necessary for telomere seeding *in vivo*.

The results presented in this paper suggest that yeast telomerase has rather high affinity and sequence specificity for the dG-rich tails of yeast telomeres *in vivo*. Why should this be necessary? At least four possibilities should be considered. First, the effective concentration of telomeric tails *in vivo* may be extremely low, for example because of masking by other proteins. Thus a telomerase with high affinity for the tail may be required just to capture the tail prior to extension. Second, the ability of telomerase to discriminate against non-telomeric tails may prevent aberrant healing of chromosomes following breakage, which could lead to large scale deletions. Third, high affinity binding may be required for negative regulation of telomerase activity. As shown in this paper and in another study (28), primers that bind tightly to yeast telomerase tend to support less extension in general, suggesting that tight binding inhibits polymerization. This inhibition may be necessary if telomerase were to bind constitutively to the telomeric end. Finally, telomerase may participate in some telomere functions other than polymerization, such as silencing or capping (16). Binding of telomerase to telomeres may thus be necessary for such additional functions.

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