

Sequence-specific and conformation-dependent binding of yeast telomerase RNA to single-stranded telomeric DNA

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ABSTRACT

Telomerase is a ribonucleoprotein reverse transcriptase responsible for the maintenance of one strand of telomere terminal repeats. The mechanisms whereby telomerase recognizes chromosomal ends are not fully characterized. Earlier studies showed that the yeast telomerase RNP could bind the dG-rich strand of yeast telomeres with high affinity and sequence specificity. Further analysis of telomerase–telomere complex formation *in vitro* as described in this report led to the following conclusions. First, telomerase binding to short DNAs is magnesium-dependent, while binding to long DNAs is magnesium-independent, consistent with the existence of more than one interaction site. Second, binding is likely to be mediated largely through the RNA subunit of telomerase (TLC1), because deproteinated TLC1 RNA also binds telomeres with high affinity and sequence specificity, and exhibits the same length and divalent cation dependence as telomerase RNP. The crucial role of RNA in binding is further supported by the ability of TLC1 transcripts synthesized *in vitro* to form stable complexes with telomeric DNA. Finally, results from deletion analysis and RNase H-mediated cleavage suggest that a specific conformation(s) of the RNA is required for stable binding, and that non-template regions of the TLC1 RNA may contribute directly or indirectly to the stability of the RNA–DNA complex.

INTRODUCTION

Telomerase is a ribonucleoprotein (RNP) that is responsible for maintaining the terminal repeats of telomeres in most organisms (1–3). It acts as an unusual reverse transcriptase, using a small segment of an integral RNA component as template for the synthesis of the dGT-rich strand of telomeres (4,5). DNA synthesis by telomerase *in vitro* is primed by oligodeoxynucleotides with telomere-like sequences. In some instances, multiple repeats can be added to the input primer in a single round of extension, despite the minimal number of

repeat units in the RNA template, implying an ability of the enzyme to undergo a translocation step without dissociating from the DNA primer (6). The telomerase complex also possesses an endonuclease activity that can cleave the starting primer under certain conditions (7–10).

Telomerase activity has been detected in a wide range of organisms including protozoa (11), yeast (8,12–14), mouse (15), *Xenopus* (16) and human (17). Genes encoding the RNA component of the enzyme complex have been cloned for several of the known telomerases, such as those of yeast and humans (18,19). Recently, some of the polypeptide components of telomerase were cloned. In particular, a yeast protein known as Est2p and homologs of Est2p in some ciliated protozoa, human (hEST2) and *Schizosaccharomyces pombe* (Trt1) were shown to be the catalytic components of the respective telomerases (12,20–24); these polypeptides exhibit significant homology to other reverse transcriptases and mutations that alter 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 copurify with *Tetrahymena* telomerase have been cloned and been shown to interact with telomerase RNA and the DNA primer, respectively (25,26). Mouse and human homologs of p80 have also been identified, and been shown to associate with the respective telomerases (27,28). These recent developments should greatly facilitate structure–function analysis of telomerase.

The mechanisms whereby telomerase recognizes the ends of chromosomes *in vivo* are not clearly understood. Telomerase from *Tetrahymena* has been shown to bind with high affinity not only to its own telomeric repeat, but also to a number of other GT-rich repeats (29). Furthermore, in this and other ciliated protozoa, even AT-rich non-telomeric sequences can efficiently support the seeding of telomeres *in vivo* and primer extension by isolated telomerase *in vitro* (30). Thus, ciliate telomerases appear not to recognize telomeres with high sequence specificity. Components of ciliate telomerase responsible for primer binding have been investigated by crosslinking and gel mobility shift experiments, and identified as the aforementioned p95 in the case of *Tetrahymena thermophila* (25,26), and as p123 and telomerase RNA in the case of *Euplotes aediculatus* (31).

In contrast to ciliated protozoa, yeast telomerase appears to bind yeast telomere repeats with high affinity and sequence

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Table 1. Sequence specificity of the RNP–DNA and RNA–DNA complexes

Oligo*	Sequence	Molar Ratio Required for 1/2 competition of RNP#	Molar Ratio Required for 1/2 competition of RNA#
TEL15	TGTGTGGTGTGTGGG	1.0	0.7
TEL15(-2)G->C	TGTGTGGTGTGTGCG	1.9	4.3
TEL15(-5)G->C	TGTGTGGTGTGTGGG	45	100
TEL15(-7)G->C	TGTGTGGTGTGTGGG	5.3	1.7
TEL15(-9)G->C	TGTGTGGTGTGTGGG	5.9	11
TEL15(-12)G->C	TGTGTGGTGTGTGGG	72	180
(TG) _n	TGTGTGGTGTGTGTGTG	1.0	0.6
(TGG) _n	TGGTGGTGGTGGTGGG	350	420
(TGGG) _n	TGGGTGGGTGGGTGGG	230	28
(TAG ₁₋₃) _n	TAGGGTAGTAGTAGGG	1300	2800
TEL19	TGTGTGGGTGTGTGGGTGT	2.3	0.5
TEL24	TGTGTGGGTGTGTGGGTGTGGG	1.0	0.4
TEL24(-12)T->A(-13)G->C	TGTGTGGGTGTGTGGGTGTGGG	3.7	0.4
TEL24(-20)T->A(-21)G->C	TGTGTGGGTGTGTGGGTGTGGG	3.2	0.6
H4CSPRA	TTTAAATGATGGAA	N.D.±	>3300
PTEL16	GTTACAGGTTACAGGG	>2500	>3300
HS1	TTAGGGTTAGGGTTAGGGTTAGGG	>1700	>3300

*TEL oligos are named according to their lengths and mutations. For example, TEL15(-3)G→C is a 15-nucleotide primer carrying a G→C mutation at the -3 position.

#Molar ratio of competitor to probe required for 50% competition of the signal observed for the RNP–TEL15 or RNA–TEL15 complex.

± Not determined.

specificity. In one study, yeast telomerase was shown not to dissociate from yeast telomeric primers following polymerization (32). Earlier work from this laboratory has shown that such high affinity binding can occur even in the absence of polymerization (33,34). In addition, yeast telomeric repeats bearing point mutations and non-yeast repeats exhibit greatly reduced affinity for the telomerase RNP. The structural determinants for this sequence-specific recognition are further analyzed in this report and shown to reside mostly in TLC1 RNA, the RNA component of *Saccharomyces cerevisiae* telomerase. De-proteinated TLC1 RNA exhibits similar telomere-binding properties to the native telomerase RNP. Comparison of the binding efficiency of telomerase RNA derived from the native RNP and from *in vitro* transcription suggests that the native conformation of the RNA is important for the RNA–DNA interactions. Furthermore, deletion analysis suggests that non-template regions of the RNA contribute either directly or indirectly to this stable interaction.

MATERIALS AND METHODS

Strain and oligodeoxynucleotides

The haploid *S.cerevisiae* strain DG338 (gift of D. Garfinkel, NCI) was used for the derivation of active telomerase. The oligodeoxynucleotides used are listed in Table 1.

Purification of *S.cerevisiae* telomerase

The derivation of whole cell extracts and the active DEAE fractions were as previously described (8,33).

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 RNP and DNA primer was monitored using a previously described gel mobility shift protocol (35). For competition assays, increasing amounts of unlabeled oligodeoxynucleotides were added to the reaction mixture prior to the addition of labeled probe and telomerase fractions. To analyze complex formation between TLC1 RNA and DNA primer, 6 µl of telomerase fraction was pretreated with 0.5 µg of proteinase K in 1% SDS at 30°C for 10 min, prior to binding and electrophoresis.

RNA analysis in native gels

To follow the 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 the separation of complexes. Following electrophoresis, the part of the gel to be analyzed for the 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 (36). The blot was then probed with a labeled TLC1 DNA fragment using standard protocols.

In vitro transcription of TLC1 RNA

The TLC1 gene was cloned by PCR amplification of yeast genomic DNA using *Pfu* polymerase (Stratagene). The forward

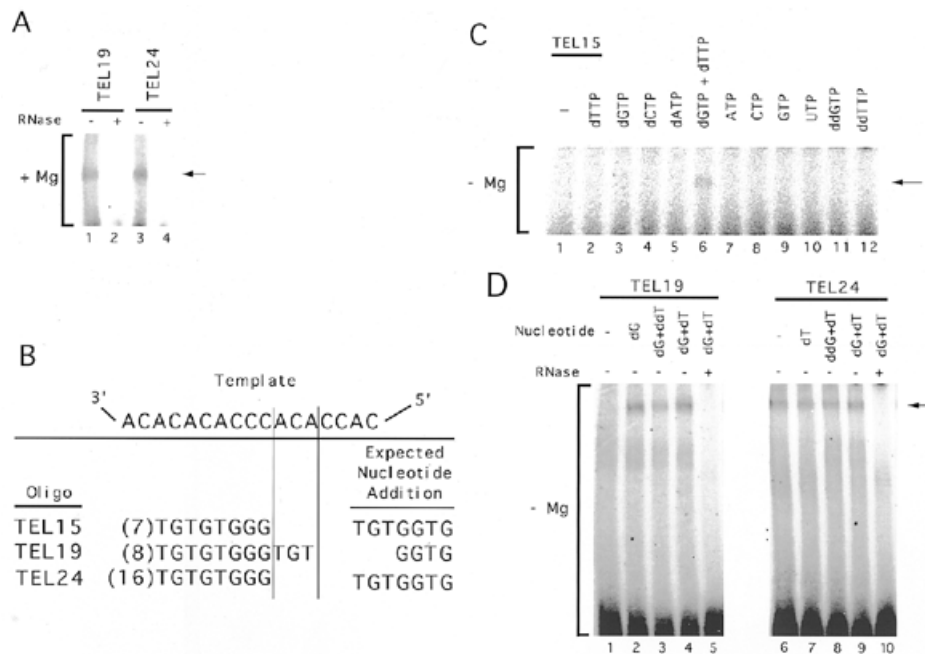


Figure 1. The effect of DNA length on the formation of telomerase–telomere complex. (A) Gel mobility shift assays were carried out using labeled TEL19 (lanes 1 and 2) or TEL24 (lanes 3 and 4) and DEAE fractions. In some cases, the fraction was pretreated with RNase A (10 ng) prior to binding and electrophoresis (lanes 2 and 4). Magnesium was included at 5 mM in both the gel and the electrophoresis buffer. (B) The optimal alignment between the template region of the TLC1 RNA and oligodeoxynucleotides TEL15, TEL19 and TEL24 is shown. The nucleotides that were expected to be added by telomerase to these oligodeoxynucleotides in a single round of extension to the end of the template are also listed. (C) Gel mobility shift assays were carried out using labeled TEL15 and DEAE fractions. Nucleotides were included at 50 μ M in some binding reactions as indicated at the top of the panel. Magnesium was omitted from both the gel and the electrophoresis buffer. (D) Gel mobility shift assays were carried out using labeled TEL19 (lanes 1–5) or TEL24 (lanes 6–10) and DEAE fractions. Nucleotides were included at 50 μ M in some binding reactions as indicated at the top of the panels. To demonstrate the specificity of the complex, the fraction was in some cases pretreated with RNase A (10 ng) prior to binding and electrophoresis (lanes 5 and 10). Magnesium was omitted from both the gel and the electrophoresis buffer.

and reverse primers used were as follows: d(GTG AGC GCG CGT AAT ACG ACT CAC TAT AGG GAA TAA AAC TAG AGA GGA AGA TAG G) and d(ACC GCT CGA GTA AAT ATT AAG AGG CAT ACC TCC G). The PCR products were cleaved with *Bss*HIII and *Xho*I enzymes, and inserted in between the *Bgl*III and *Xho*I sites of the pET16b plasmid to generate pET-TLC1. The fidelity of the PCR reaction was confirmed by sequencing of the resulting plasmid. TLC1 RNA was synthesized from pET-TLC1 cleaved with various restriction enzymes using a T7 Riboprobe system (Promega). For synthesis of full-length TLC1, the template was cleaved with *Xho*I, which resulted in the addition of five nucleotides (CTCGA) to the natural 3' end of the RNA. All other RNAs synthesized *in vitro* contained only TLC1 sequences.

Antisense oligodeoxynucleotide- and RNase H-mediated cleavage of TLC1 RNA

Approximately 2 ng of TLC1 RNAs isolated from fractions or derived from *in vitro* transcription were incubated with 4 ng of an antisense oligodeoxynucleotide d(TGTGTGTGGGTGTGGTG) in RNase H buffer (USB) at 37°C for 5 min, and one-half of the mixture treated with 2.5 U of RNase H for 10 min. The mixtures were further digested with proteinase K, extracted with phenol/chloroform/isoamyl alcohol, and subjected to ethanol precipitation. The cleaved RNAs were then fractionated in a 6% denaturing polyacrylamide gel, electroblotted onto Nylon membrane (Hybond N, Amersham) at 150 mA for

3 h in 0.5 \times TBE buffer, and visualized by probing with 32 P-labeled DNA corresponding to the full-length TLC1 gene (37).

RESULTS

The effects of length and divalent cation on the formation of telomerase–primer complex

Using a gel mobility shift assay, we have previously shown that the yeast telomerase RNP binds single-stranded telomeres with high affinity and sequence specificity (33,34). Interestingly, detection of the telomerase–telomere complex in the absence of polymerization was found to require the presence of magnesium or some other divalent cation in the gel and electrophoresis buffer. However, an earlier study by Prescott and Blackburn (32) indicates that a stable complex can be observed between telomerase and primers following polymerization in the absence of magnesium. This discrepancy can be rationalized in one of three ways. First, the interaction between telomerase and primer may be qualitatively different pre- and post-polymerization. Second, the presence of nucleotides may allosterically alter the binding of telomerase to telomeres. Finally, because the oligodeoxynucleotides are lengthened by telomerase following extension, the differential divalent cation requirement may simply be due to a difference in the length of the primer. To distinguish between the possibilities, we tested complex formation between telomerase and primers of varying

length in the presence of various nucleotides, using either magnesium-plus or magnesium-minus buffer systems.

Three dG-rich telomere-like primers were utilized for these assays: TEL15, TEL19 and TEL24. (The numbers in the names designate the lengths of the oligodeoxynucleotides; see Materials and Methods and Table 1 for explanation.) The optimal alignment between the TLC1 RNA template region and the three oligodeoxynucleotides that allows primer extension is shown in Figure 1B, along with the sequences that are expected to be added to the three primers. In the presence of magnesium, telomerase could form stable complexes with all three primers (34; Fig. 1A). In the absence of magnesium, as shown earlier (34) and in Figure 1C (lane 1), TEL15 was unable to interact stably with telomerase. However, the addition of both dGTP and dTTP to the binding mixture, allowing in principle the addition of seven deoxyribonucleotides, enabled one to detect the complex (lane 6). Either nucleotide alone was insufficient (lanes 2 and 3). The addition of other deoxyribonucleotides or ribonucleotides also failed to stimulate complex formation (lanes 4, 5, 7–12). TEL19 was also unable to bind telomerase stably in the absence of magnesium (Fig. 1D, lane 1). However, in the case of TEL19, the inclusion of dGTP alone, allowing in principle the addition of just two nucleotides to the primer, was sufficient to stabilize the complex (lane 2). The further inclusion of ddTTP or dTTP had no effect (lanes 3 and 4). When the TEL24 primer was tested in the magnesium-minus buffer system, no requirement for any nucleotide triphosphate was evident (lanes 6–9). These results taken together indicate that the formation of a magnesium-independent complex may simply be a function of the length of the oligodeoxynucleotide, rather than a consequence of the act of polymerization or the direct effects of specific nucleotides. The minimal length requirement for the formation of the magnesium-independent complex is estimated to be 21 nucleotides.

Deproteinized yeast telomerase can form a stable complex with the dG-rich strand of yeast telomeres

To gain insight into the subunits of telomerase responsible for sequence-specific recognition, we subjected the telomerase fractions to either proteinase K or RNase A pre-treatment. Consistent with earlier studies, the addition of RNase A prior to binding and electrophoresis was found to abolish complex formation (Fig. 2A, lane 3). On the other hand, the addition of proteinase K (0.5 μ g) and SDS (1%) resulted in the disappearance of the original complex, but the appearance of a new complex of slightly greater mobility (Fig. 2A, lane 2). Two lines of evidence suggest that the new complex is protein-free and represents the binding of naked TLC1 RNA to telomere oligodeoxynucleotide. First, no further changes in mobility were visualized using telomerase fractions that had been subjected to extraction with phenol and precipitation with ethanol (Fig. 2B, compare lanes 3 and 4 with lane 2). Second, judging by northern blotting experiments (Fig. 2B, lanes 5 and 6), the complex co-migrated with de-proteinized TLC1 RNA in the same native gel.

Similar sequence specificity for the formation of the RNA–DNA and the RNP–DNA complexes

To determine if protein-free TLC1 RNA exhibits similar telomere recognition properties as telomerase RNP, we analyzed the binding specificity of the RNA in a series of competition gel mobility shift experiments. Complex formation between a

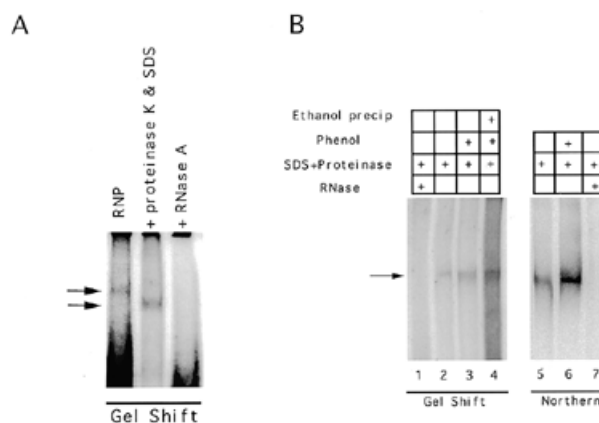


Figure 2. De-proteinized TLC1 RNA can form a stable complex with single-stranded yeast telomeric DNA. (A) Gel mobility shift assays were carried out using labeled TEL24 and DEAE fractions. The fraction was either pretreated with SDS (1%) and proteinase K (0.5 μ g) or pretreated with RNase A (10 ng) prior to binding and electrophoresis as indicated at the top of the figure. Magnesium was included at 5 mM in both the gel and the electrophoresis buffer. (B) In the left panel, gel mobility shift assays were carried out using labeled TEL15 and pretreated fractions. Magnesium was included at 5 mM in both the gel and the electrophoresis buffer. The treatment for each fraction was indicated at the top of the figure. In the right panel, the treated fractions were directly electrophoresed into the same gel without the addition of labeled TEL15. Following electrophoresis, the RNA was denatured *in situ*, transferred to nylon membrane, and probed with labeled TLC1 DNA.

labeled yeast telomere oligodeoxynucleotide and de-proteinized telomerase was measured in the presence of increasing concentrations of various unlabeled oligodeoxynucleotides such that their relative affinities can be estimated from the degree of competition (Fig. 3). Similar to the telomerase RNP, protein-free TLC1 RNA bound preferentially to single-stranded dG-rich oligodeoxynucleotide: two canonical yeast telomeric repeat (TEL15 and TEL24) competed well for complex formation, whereas three unrelated oligodeoxynucleotides (H4CSPRA, PTEL16 and HS1) failed to compete. Additional oligodeoxynucleotides that carried point mutations or alternative repeats were tested and the amount of each oligodeoxynucleotide required for 1/2 competition of the RNA–DNA complex determined and tabulated in Table 1. The relative affinities of these oligodeoxynucleotides for the telomerase RNP as determined in earlier studies (33,34) were also listed for comparison. The results indicate that protein-free TLC1 RNA has a sequence specificity that is very close to that of the telomerase RNP. For example, among the 15-nucleotide series of primers, point mutations at the –5 and –12 positions had the greatest effects on the binding of both the RNA and the RNP. Those point mutations that had a lesser effect on RNP binding also had a lesser effect on RNA binding (mutations at the –2, –7 and –9 positions). In addition, for both the RNA and the RNP, the (TG)_n and the (TGGG)_n oligodeoxynucleotides exhibited greater affinity than the (TGG)_n and (TAG)_{1,3}_n oligodeoxynucleotides.

Similar length and divalent cation requirements for the formation of the RNA–DNA and the RNP–DNA complexes

Two other similarities between the RNA–DNA and the RNP–DNA complex point to similar mechanisms of recognition:

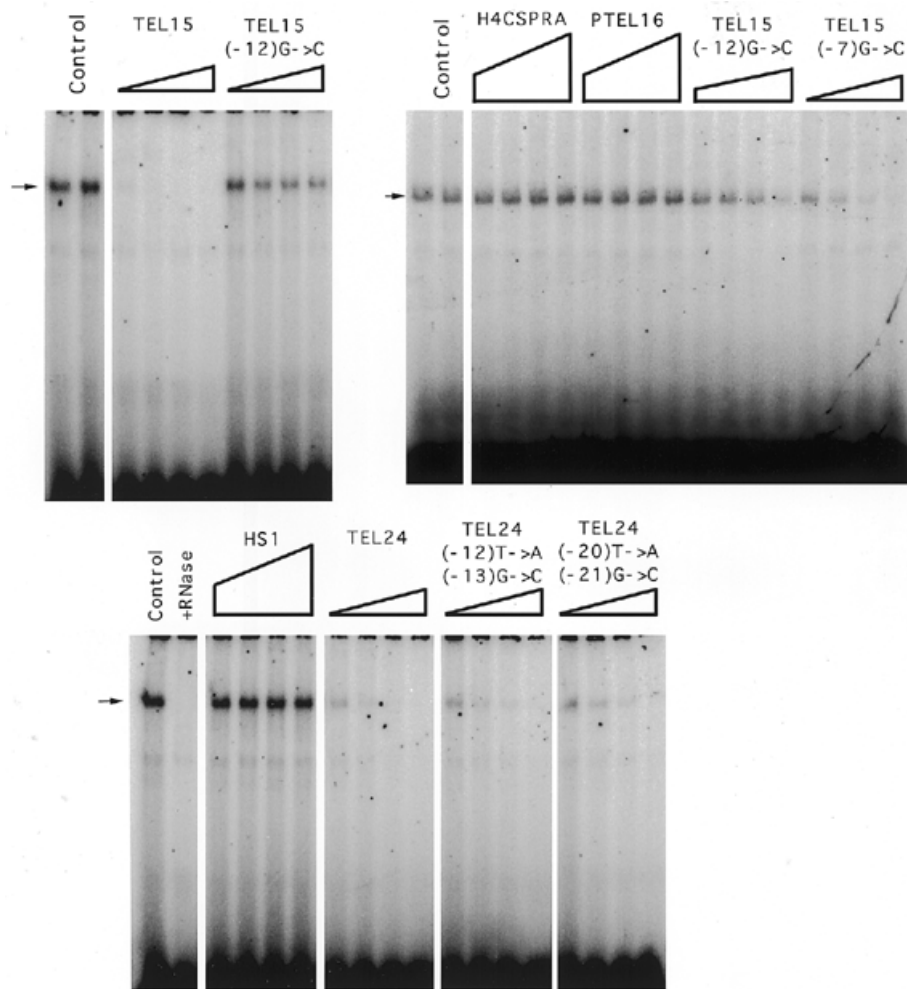


Figure 3. Sequence-specific recognition of telomeric DNA by de-proteinated TLC1 RNA. Gel mobility shift assays were carried out using labeled TEL15 as probe and telomerase fractions that had been pretreated with SDS and proteinase K. Magnesium was included at 5 mM in both the gel and the electrophoresis buffer. Increasing amounts of various unlabeled oligodeoxynucleotides were included as competitors in some assays as indicated at the top of the panels. The molar ratio of unlabeled to labeled oligodeoxynucleotides were as follows: for TEL15, TEL15(-7)G→C; TEL24, TEL24(-12)T→A(-13)G→C, TEL24(-20)T→A(-21)G→C: 5-, 15-, 45-, 135-fold (left to right); for TEL15(-12)G→C: 25-, 75-, 225-, 675-fold (left to right); for H4CSPRA, PTEL16 and HS1: 100-, 300-, 900-, 2700-fold (left to right). The control reactions contained no cold competitor oligodeoxynucleotides and the +RNase reaction used a telomerase fraction that had been pretreated with RNase A before the addition of proteinase K and SDS.

both complexes are resistant to high salt (data not shown), and for certain oligodeoxynucleotides, the stability of both requires the presence of divalent metal ions. As shown in Figure 4, in the presence of magnesium, de-proteinated TLC1 RNA can stably interact with TEL15, TEL19 and TEL24. A point mutation at the -12 position of TEL15 abolished binding, consistent with the competition study (Fig. 4 and Table 1). Most remarkably, in the absence of magnesium only TEL24 was capable of stable binding to the RNA, as has been found for the RNP.

TLC1 RNA derived from *in vitro* transcription can form a stable complex with single-stranded yeast telomeres

To begin to dissect the portions of yeast telomerase RNA required for stable interaction with single-stranded telomeres, we cloned the TLC1 RNA gene behind a bacteriophage T7 promoter, and synthesized full-length RNA *in vitro*. The RNA was tested for its ability to bind telomeres in the standard gel mobility shift assay using the magnesium-plus buffer system.

Interestingly, very high concentrations of the *in vitro* transcribed RNA were needed to observe a specific complex. In the experiment shown in Figure 5, ~500-fold more *in vitro* transcribed RNA was used than the native telomerase RNA. Yet the signal for the former was only two to three times more than the latter (compare lanes 1 and 7), indicating that the RNA transcribed *in vitro* was at least 200-fold less efficient at telomere binding, possibly because of aberrant folding. Various strategies have been used in an attempt to improve the efficiency of binding by RNA synthesized *in vitro*, thus far without much success.

Evidence for interaction between non-template region of the telomerase RNA and DNA primer

A preliminary truncation analysis was carried out to de-limit the region of telomerase RNA required for telomere binding. The T7 promoter-containing template was truncated by restriction enzymes at various positions, and used for the synthesis of TLC1 RNA missing increasing amounts of 3' end sequences.

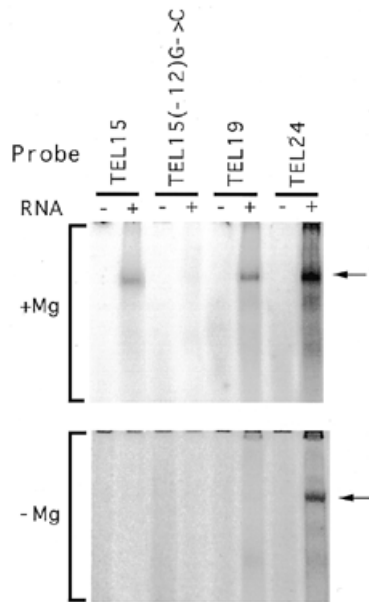


Figure 4. The effects of length and magnesium on the formation of the RNA-DNA complex. Gel mobility shift assays were carried out using labeled oligodeoxynucleotides as probes (as indicated at the top of the figure) and telomerase fractions that had been pretreated with SDS and proteinase K. The fraction was omitted in the control reactions (-). In the top panel, magnesium was included at 5 mM in both the gel and the electrophoresis buffer. In the bottom panel, magnesium was omitted from both the gel and the electrophoresis buffer.

As shown in Figure 5A and B, deletion of 384 residues resulted in a 3-fold increase in binding (1-917, lane 2), whereas deletion of 639 or 671 residues (1-662 and 1-630, lanes 3 and 4) greatly reduced specific complex formation. However, a significant level of background smear was observed with all of these deletions, consistent with unstable binding and dissociation during electrophoresis. When the deletion removed the template portion of telomerase RNA (residues 468-484), even the background smear was abolished (1-450 and 1-389, lanes 5 and 6). We surmise that perhaps the template portion of TLC1 is indispensable for stable binding, while sequences between residues 662 and 917 contribute either directly or indirectly to complex stability.

To begin to analyze the conformation of the RNAs used in binding studies, I determined the accessibility of the template region to cleavage by RNase H in the presence of a complementary oligodeoxynucleotide. TLC1 RNAs derived from telomerase fractions or generated by *in vitro* transcription were incubated with an antisense DNA complementary to the entire 17-nucleotide template region, subjected to cleavage by RNase H and fractionated by denaturing polyacrylamide gels. Following transfer to nylon membranes, the TLC1 RNAs were detected by hybridization with a specific probe. As expected, the template region of the TLC1 RNA derived from telomerase fractions was fully accessible (Fig. 5C, lanes 1 and 2). Both the 1-1301 and the 1-917 transcripts were also completely processed by RNase H, indicating that their template regions were exposed (Fig. 5C, lanes 3-6). The greatly reduced binding efficiency of the 1-1301 and 1-917 transcripts relative to TLC1 RNA

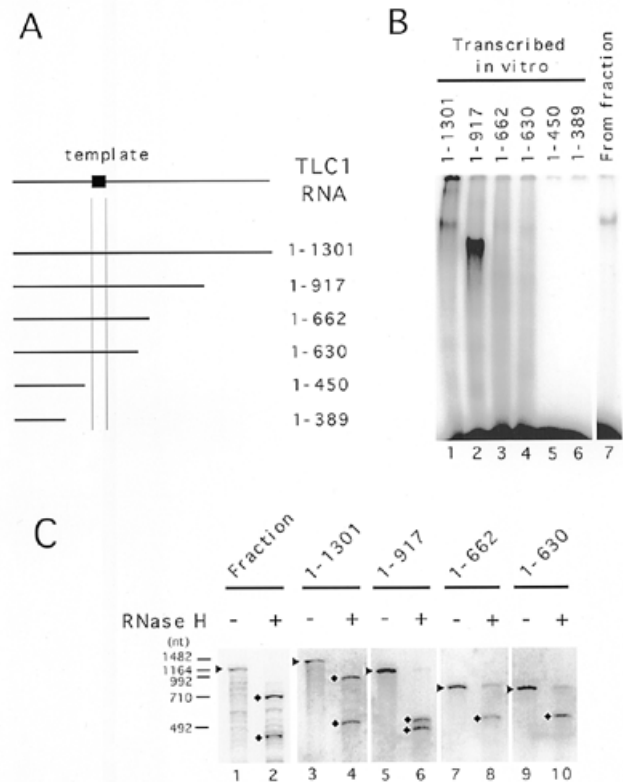


Figure 5. Formation of complex between RNA transcribed *in vitro* and telomeric DNA. (A) Schematic diagram of TLC1 transcripts synthesized *in vitro* by bacteriophage T7 RNA polymerase. The location of the template region is indicated by a black box. (B) Gel mobility shift assays were carried out using labeled TEL15 and RNA transcribed *in vitro* (lanes 1-6) or derived from fraction (lane 7) as indicated at the top of the figure. Magnesium was included at 5 mM in both the gel and the electrophoresis buffer. The residues of TLC1 RNA retained in the *in vitro* transcripts were explicated at the top of the figure. The templates for these transcripts were prepared by cleaving PET-TLC1 with the following enzymes: 1-1301, *Xho*I; 1-917, *Afl*III; 1-662, *Hpa*I; 1-630, *Bln*I; 1-450, *Nco*I; 1-389, *Xmn*I. The amounts of TLC1 RNA used for binding were as follows: 1-1301, 300 fmol; 1-917, 300 fmol; 1-662, 550 fmol; 1-630, 550 fmol; 1-430, 550 fmol; 1-389, 550 fmol; fraction, 0.6 fmol. (C) TLC1 RNAs were treated with antisense oligodeoxynucleotides, further incubated in the absence (-) or presence (+) of RNase H, and the resulting products analyzed by northern hybridization. The identity of the RNA used was indicated at the top of the panels. Full-length RNA or transcripts are indicated by filled triangles, and the specific cleavage products by filled diamonds. The positions of labeled DNA molecular weight standards are indicated to the left of the panel.

derived from telomerase fraction cannot therefore be explained by the inability of the 1-1301 transcript to base-pair with primer DNA. Some other aspect of the RNA conformation in addition to an exposed template region must also be important for stable binding.

In contrast to the 1-1301 and 1-917 transcripts, a significant fraction (~30-45%) of the 1-662 and 1-630 transcripts were not cleaved, consistent with the existence of conformers whose templates were not exposed (Fig. 5C, lanes 7-10). This result suggests that sequences between residue 662 and 917 of TLC1 contribute to the exposure of the TLC1 template, probably by affecting the overall folding of the RNA.

The size and the amount of the cleavage products appear to accurately reflect the exposure of the template region. The fragments derived from all of the RNAs were consistent with cleavage at the template region (residues 468–484). For example, cleavage of transcript 1–917 resulted in fragments of ~480 and 430 nucleotides, precisely as expected (lanes 5 and 6). In addition, the amount of RNase H used was not limiting; the addition of more RNase H had no effect on the experimental results (data not shown). We also note that the 1–1301 transcript (as well as its cleavage products) was significantly longer than TLC1 RNA derived from telomerase fraction (compare lanes 1 and 3, and lanes 2 and 4). This is consistent with an earlier report showing that the polyadenylation sites of the TLC1 RNA are clustered around nucleotide residue 1255 (37). Whether the 3' end of the RNA in telomerase RNP corresponds to one or more of the polyadenylation sites remains to be determined.

DISCUSSION

Perhaps the most significant observation made in this report is that the RNA component of yeast telomerase is largely responsible for high affinity and sequence-specific recognition of telomeres by the RNP. This result is consistent with earlier reports of protein-independent binding of telomerase RNA to telomeric DNA, which came from studies of the enzyme from *Euplotes* (31,38). Other studies from ciliates implicate both the RNA and protein components of telomerase in telomere recognition (23,25,29,31). In particular, the p95 subunit of *Tetrahymena* telomerase was shown to mediate RNA-independent recognition of single-stranded telomeres with high affinity, and treatment of *Tetrahymena* telomerase with proteinase K was reported to abolish complex formation entirely. In addition, the p123 subunit of *Euplotes* telomerase can be specifically crosslinked to telomeric primers, consistent with a role in telomere binding. Clearly, our results do not rule out the participation of yeast telomerase proteins in stable telomere binding. However, there is no obvious appearance of a faster moving complex in the gel mobility shift assay when yeast telomerase fraction was pretreated with RNase A, suggesting that the protein subunits are unable to bind telomeric DNA stably on their own.

The mechanism whereby primer length modulates magnesium requirement in binding is not understood. An attractive hypothesis is that longer primers possess greater potential for base pairing with the template, which interaction does not depend upon magnesium. Alignment of the TEL15, TEL19 and TEL24 oligodeoxynucleotides with the template region of TLC1 RNA indicates that they can form a maximum of a 9, 12 and 13 bp continuous hybrid, respectively. [In Fig. 1B, the optimal alignments that allow primer extension are shown. However, to maximize the length of the hybrids, TEL19 and TEL24 can be positioned further toward to 5' end of the RNA template such that the more 5' d(GGG)s of the primer form base pairs with the (CCC) residues of the template. In this other configuration, telomerase would not be capable of extending the two primers.] If the continuity of the hybrid is allowed to be interrupted, these oligodeoxynucleotides can form base pairs with 9, 13, 14 nucleotides of the template, respectively. Thus the longer oligodeoxynucleotides tested in our experiments can indeed potentially form more extensive hybrids with the telomerase RNA. In the absence of sufficient base pairing, perhaps a

second interaction mediated in part by magnesium is required for stable binding. Consistent with this conjecture, crosslinking studies using *Euplotes* telomerase have revealed close proximity between non-template telomerase RNA residues and primer residues (31). Similar studies on the yeast telomerase RNA–DNA complex may begin to shed light on the molecular basis of interactions away from the template region.

At least two aspects of the RNA conformation appear necessary for stable complex formation. First, the template region must be exposed. Transcripts missing residues from 663 to 917 exhibited simultaneously reduced accessibility of their template region and reduced ability to form stable RNA–DNA complexes. Thus, this ~250-nucleotide region may play an important role in the function of TLC1 RNA indirectly by maintaining the exposure of the template region. In addition, some other as yet undefined aspect of the conformation must also be important; full-length TLC1 RNA synthesized *in vitro*, despite the exposure of its template region as determined by RNase H cleavage, is much less efficient at complex formation than native TLC1. Further structural and functional characterization of the TLC1 RNA will be necessary to define this other feature(s).

Our observation that the RNA component of telomerase is largely responsible for mediating stable interaction of the RNP with single-stranded telomeric DNA provides an experimental approach for addressing the physiological function of this stable interaction, which is currently unknown. It has been suggested that by stably interacting with telomeres *in vivo*, telomerase can serve a protective 'capping' function (32,39). Alternatively, if telomerase is indeed a structural component of telomeres, it may play a role in telomere position effect or telomere length regulation (18,33). By further defining the structural and mechanistic basis for the stable interaction between telomerase RNA and telomeric DNA, one may be able to identify mutations within the RNA that will selectively disrupt stable interaction. By incorporating such mutations into the TLC1 gene *in vivo*, one will then be able to determine the physiological functions, if any, of the stable interaction between telomerase and telomeres.

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