

Yeast telomerase is specialized for C/A-rich RNA templates

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ABSTRACT

Telomeres, the protective caps of eukaryotic chromosomes, are maintained by the enzyme telomerase. This telomere-specific reverse transcriptase (RT) uses a small region of its RNA subunit as template to synthesize telomeric DNA, which is generally G/T rich in the strand that contains the 3' end. To further our understanding of why telomeres are usually G/T rich, we screened *Saccharomyces cerevisiae* telomerase RNA (*TLC1*) libraries with randomized template sequences for complementation of a *tlc1* deletion and decapping of existing telomeres. Surprisingly, the vast majority of the 60 000 different mutant telomerase templates tested showed no activity *in vivo*. This deficiency was not due to impaired assembly with the catalytic subunit (Est2p) nor could it be alleviated by enforced telomerase recruitment to the telomeres. Rather, the mutant templates reduced the nucleotide addition processivity of telomerase. The functional RNA template sequences recovered in our screens preferentially contained two or more consecutive rC nucleotides, reminiscent of the wild-type template. Thus, in contrast to retroviral RTs that can reverse transcribe any RNA sequence into DNA, the budding yeast telomerase RT is specialized for its C-rich RNA template.

INTRODUCTION

In most organisms, telomere sequences are rich in guanine and thymine nucleotides in the DNA strand that runs 5' to 3' towards the chromosome end (1). Furthermore, telomere repeats usually contain runs of several adjacent deoxyguanosine nucleotides. This sequence feature allows the formation of a stable DNA secondary structure, the G-quadruplex, *in vitro* [reviewed by Williamson (2)]. The high degree of telomere sequence conservation suggests some functional importance. However, the precise molecular events in which sequence-specific functions of the telomere are involved have not been identified so far.

The essential function of telomeres is to prevent chromosome end-to-end fusions and extensive nucleolytic degradation [reviewed by McEachern *et al.* (3)]. Loss of end protection could be provoked by changes in the telomeric DNA sequence (4–7). Presumably, the mutant telomere sequences interfere with the formation of an essential telomeric chromatin and DNA structures such as the G-quadruplex. G-rich DNA secondary structures have also been proposed to participate in the telomerase reaction cycle (8). Specifically, the folding of newly synthesized telomeric repeats into G–G hairpins or G-quadruplex structures may facilitate the product dissociation and translocation steps by lowering the energy difference between the extended, base-paired telomere–template hybrid and the dissociated individual strands (9,10).

Whether the telomerase RNA template participates in a sequence-specific manner in the reaction or serves only as a passive template has been discussed controversially in the past. Several completely non-telomeric RNA templates can be reverse transcribed by *Tetrahymena thermophila* telomerase (11), and limited incorporation of mutant telomere sequences specified by ectopically expressed mutant telomerase RNA templates occurred in human tumor cells (12,13). On the other hand, changes in the product dissociation pattern, reduced fidelity and lowered processivity have been described for template mutant *Tetrahymena* telomerases (14–16). A template mutant yeast telomerase was inactive in mutant enzyme homomultimers but active in wild-type/mutant heteromultimers (17,18). So far, no general rule for the RNA template's role in the telomerase reaction has emerged from the published experiments.

In this study, we chose a genetic approach to define the template sequence requirements of budding yeast telomerase and gain insight into the reasons why telomeres are generally G/T rich. A telomerase RNA template library, in which 10 of the 16 templating nucleotides were randomized, was screened for complementation of a *tlc1* deletion and, in a separate screen, for the induction of growth arrest. This unbiased analysis of a large number of template sequences revealed that telomerase can reverse transcribe only a minor fraction of all possible templates *in vivo*. Mutant template RNAs that complemented a *tlc1* deletion preferentially contained at least two consecutive rC nucleotides, similar to the central part of wild-type *TLC1*. To verify the functional importance of this sequence, we constructed several telomerase RNA template

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libraries with five or six randomized template nucleotides, thus spanning the entire RNA template region. The number of complementing templates was especially low in a library where the central ⁴⁷⁷CCCAC⁴⁷⁵ template sequence was randomized, emphasizing the importance of this region. The deficiency conveyed by the template mutations was a reduced telomerase nucleotide addition processivity, indicating that the *Saccharomyces cerevisiae* TERT enzyme has a functional dependence on the C/A-rich RNA template sequence.

MATERIALS AND METHODS

Library construction

Mutagenesis of *TLC1* was carried out as previously described (19) by ligating a PCR product obtained with oligonucleotide primers carrying random nucleotides at the desired positions (sequence of the mutagenesis primer: 5'-TAATTATCAT-GAGAAGCCTACCATCACCACCCACACACAATGTTCAG-3'; the underlined sequence corresponds to the template region and was changed according to the desired library design) with a plasmid vector containing the rest of the *TLC1* gene. The ligation products were transformed into competent *Escherichia coli* cells for amplification. For the short libraries (Library₄₈₃₋₄₇₈, Library₄₇₇₋₄₇₃ and Library₄₇₂₋₄₆₈), the number of transformants was considerably higher than the theoretical complexity of these libraries. For Library₄₈₀₋₄₇₁, approximately 200 000 bacterial transformants were obtained, which corresponds to one-fifth of the theoretical complexity. Individual clones from each library were sequenced to confirm randomization of each nucleotide position in the desired region.

Screen for complementation of *tlc1-Δ*

YKF19 [*Mat a ade2 his3-11 can1-Δ leu2 trp1 ura3-52 DIA5-1 (ADE2 telomere VR) tlc1::HIS3 rad52::LEU2*] was re-streaked progressively until senescence. The second from last streak was used to inoculate a liquid culture for transformation, and the cells were incubated until no further growth was detectable. The transformation efficiency was determined by counting the number of colonies obtained with wild-type *TLC1* (pSD107). Control transformations with the empty vector (pRS314) gave rise to no or very few colonies. Colonies obtained upon transformation with the library plasmids were re-streaked at least twice. The *tlc1* template region from plasmid library-harboring colonies was PCR amplified using the two oligonucleotides 5'-*TLC1*_long 5'-GGCCCGGGAATAAACTAGAGAGGAAGATAGG-3' and 3'-*TLC1*_short 5'-GGCCCGGACAGTGTTCAGAAAAAATACTAGG-3'. The PCR product was digested with *NcoI* to exclude contaminating wild-type plasmids, which were present in Library₄₈₀₋₄₇₁ at a frequency of 0.5%. The template region was sequenced either directly from the PCR product or from the plasmid after recovery in *E.coli*.

Screen for growth arrest

Yeast strain YKF20 [*Mat a ade2 his3-11 can-1Δ leu2 trp1 ura3-52 DIA5-1 (ADE2 telomere VR) tlc1::HIS3 pTLC1-URA3*] was transformed with Library₄₈₀₋₄₇₁ and grown under conditions that selected for both the wild-type *pTLC1-URA3* and the mutant *tlc1* from Library₄₈₀₋₄₇₁. The colonies were

replica-plated onto 5-fluoro-orotic acid (FOA)-Trp medium to select for loss of the plasmid containing wild-type *TLC1* but retention of the mutant *tlc1* plasmid. The library plasmids were recovered from candidate *TLC1/tlc1* colonies and re-transformed into a *tlc1-Δ* yeast strain well before the onset of senescence. Growth arrest-inducing *tlc1* alleles were identified by reduced transformant colony size and number relative to pRS314.

IP-RT-PCR experiments

Yeast strain YKF103 (*Mat a ura3-52 ade2-101 lys2-Δ1 trp1-Δ1 his3-Δ200/CF⁺ ProteinA-EST2*) (20) was transformed with either pKF5 (19) or Library₄₈₀₋₄₇₁. Individual colonies were picked and 10 ml cultures were grown to an OD₆₀₀ of 0.3–0.5. The cells were harvested, washed and lysed by bead bashing in 300 μl of immunoprecipitation (IP) buffer high salt [10 mM Tris-HCl pH 7.5, 150 mM NaCl, 150 mM KCl, 1 mM MgCl₂, 1 mM phenylmethylsulfonylfluoride, 0.1 mM dithiothreitol (DTT), 10% glycerol, 0.1% Tween-20, 0.1% NP-40, 0.1 U/μl of RNase inhibitor]. The extracts were cleared by centrifugation, and 500 μg of total protein were incubated with 50 μl of a 50% slurry of rabbit serum agarose beads (Sigma) to bind the protein A-tagged Est2p and 20 U of DNase I (Roche Molecular Biochemicals) for 120 min at 4°C. The beads were washed twice with 1 ml of IP buffer high salt, once with 1 ml of IP buffer low salt (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.1 mM DTT, 10% glycerol) and resuspended in 30 μl of IP buffer low salt. The reverse transcriptase reactions were carried out with Superscript II RT (Life Technologies) according to the manufacturer's instructions using 10 μl of the resuspended beads as template and oligo 3'-*TLC1*_short (see above) to prime cDNA synthesis. PCR amplification and *NcoI* digestion were performed as described above.

Telomere length analysis

Telomere length was analyzed by telomere-PCR (21) or Southern blotting. Telomere PCR products were separated on 3% agarose gels and the size was determined using AIDA image quantification software (Fuji). For Southern blotting, restriction fragments were separated on 0.7% agarose gels, transferred to nylon membrane and subsequently hybridized with a Y'-probe (22) and a probe for the 1 kb DNA size standard (Life Technologies). Autoradiographs were acquired with a Fuji BAS PhosphorImager and analyzed with AIDA software (Fuji).

Telomerase assays

Telomerase extracts were prepared and reactions performed as described (19,23). The amount of *TLC1* RNA in each preparation was determined by northern hybridization, and equal amounts were used in the reactions. For the preparation without *TLC1* RNA (empty vector), the same amount of protein as for the wild-type preparation was employed.

RESULTS

Functional telomerase RNA templates are highly enriched in C and A nucleotides

To increase the likelihood of mutant telomerases adding multiple repeats to the telomeres, we randomized only the

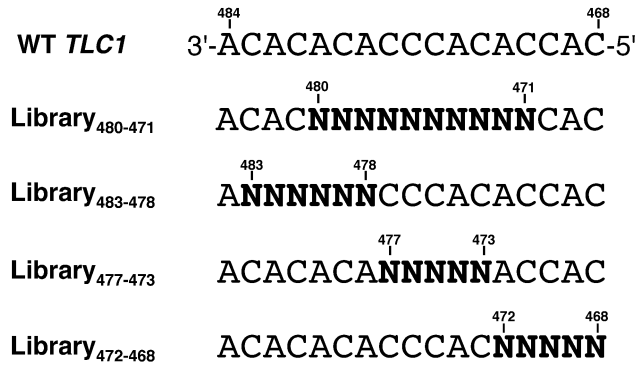


Figure 1. Schematic representation of the different *TLC1* template libraries used in this study. All telomerase RNA template sequences in the figures and text of this manuscript are written in the 3' to 5' direction, reflecting the order in which the template nucleotides are reverse transcribed by the telomerase reverse transcriptase.

central 10 nt of the wild-type *TLC1* template region (Library₄₈₀₋₄₇₁, Fig. 1). This library design should enable all mutant telomerases to base pair to the existing wild-type telomeres. In addition, the conservation of 3 nt at either end of the template should allow re-alignment of the telomeres after reverse transcription of a mutant template up to the template 5' boundary.

To select for templates that complemented a deletion of the *TLC1* gene, we transformed YKF19 (*tlc1-Δ rad52-Δ*) with Library₄₈₀₋₄₇₁ as the cells underwent senescence. The colonies obtained were re-streaked at least twice before further analysis. Out of an estimated 60 000 transformants (see Materials and Methods), 40 different mutant templates complemented the *tlc1* deletion (Table 1). Two template mutations were isolated twice (23 + 24 and 72 + 78), and one template mutation was isolated three times independently (4 + 17 + 33). Five mutant templates were shorter than the wild-type *TLC1* template region and were not taken into account in deriving the consensus sequence. Most telomerase RNA template mutations that complemented the *tlc1* deletion nevertheless resulted in slow growth, various degrees of temperature sensitivity and short telomeres. Sequence comparison of complementing full-length mutant templates revealed little similarity in positions close to the template 3' boundary, while the region close to the template 5' boundary showed a strong bias for C and A as templating nucleotides (Fig. 2, nucleotide composition over mutant region different from 25% each: $P > 99.9$, χ^2 analysis). This sequence bias was not present in the library before the screen (data not shown).

The sequence 3'-CCCCA-5' (indicated below the bar graph) is proposed as a consensus since these nucleotides are present in more than half of the template sequences at the respective positions. Together with the invariant positions due to the library design, this suggested that the sequence 3'-CCC-CACAC-5', reminiscent of the central portion in the wild-type *TLC1* template sequence, can fulfill the sequence-specific requirements for *S.cerevisiae* telomere maintenance. Since this consensus represents the most frequent nucleotide at each position but not the most frequent template sequence, the full consensus is found in only one of the mutant templates. The slightly shorter sequence 3'-CCCACAC-5' is present in six

Table 1. Complementing templates from Library₄₈₀₋₄₇₁

pLib-	template sequence (3' to 5')	telomere length (nt TG _{1,3})	23°C	25°C	30°C	36°C
WT	CACACACCCACACCAC	280	++	++	++	++
1	CACAAACCCGCAACAC	n.d.	n.d.	n.d.	+	n.d.
3	CACCGUACCCACCAC	n.d.	n.d.	n.d.	+	n.d.
4	CACCUAAACCACACAC	n.d.	n.d.	n.d.	+/-	n.d.
7	CACGUUAUCCACCAC	130	+	+	+/-	-
9	CACCGCAUACCCGCAC	170	++	+/-	+/-	++
11	CACCACAGCCCGCAC	130	+	+	+/-	-
14	CACCGCGUCCCAACAC	150	+	+/-	-	-
17	CACCUAAACCACACAC	230	++	++	++	++
22	CACAUCGACCCUACAC	140	+	+/-	-	-
23	CACCGCAAGACCACAC	200	++	++	++	++
24	CACCGCAAGACCACAC	140	+	+	+/-	-
28	CACCACAGCCCGCAC	150	+	+/-	-	-
29	CACCACAGCCCGCAC	160	+	+	+/-	-
33	CACCUAAACCACACAC	310	++	++	++	++
47	CACAUUAACCACACAC	170	+	n.d.	n.d.	n.d.
48	CACACCAAGGAGACAC	140	+	n.d.	n.d.	n.d.
50	CACUAAACAGGCACAC	150	+	n.d.	n.d.	n.d.
52	CACGGUUCAGUCCAC	130	+	+	+	+
54	CACAUCUGCGGCACAC	150	+	+	+/-	-
55	CACAUAAUACCCACAC	270	+	+	+	+/-
56	CACUGUAACAGGCAC	180	+	+	+/-	-
59	CACGAUGCACACGCAC	160	++	+	+	+
60	CACACUACGGCACAC	150	+	+	++	+
61	CACUUAAGUCCACAC	150	+/-	+/-	+/-	-
62	CACGACUCUCCACAC	n.d.	+/-	+/-	+/-	-
63	CACCGCGCCCCACAC	230	++	++	++	-
65	CACAAACACAUAACAC	180	+/-	+/-	+/-	-
66	CACAUCGUCACCACAC	n.d.	+	+	++	-
67	CACCGAAUACACACAC	210	+	+	+	-
68	CACUGGUCACACACAC	190	+	+	++	-
70	CACACAGACCCAC	180	+	+	++	+/-
71	CACUACUCCACCACAC	n.d.	++	++	+	+/-
72	CACUCUCUACCCACAC	180	+	+	+	-
73	CACGACCAAGCGACAC	180	+/-	+/-	+/-	-
74	CACGGCGGUCGAGCAC	180	+/-	+/-	+/-	-
76	CACGGGUCCACAACAC	160	+	+	+	-
78	CACUCUCUACCCACAC	160	+	+	+	-
79	CACAAAGCUAAACCAC	160	+/-	+/-	+/-	-
80	CACCACAACAUAACAC	150	+/-	+/-	+/-	+/-
81	CACUGCUAUCCACAC	n.d.	+/-	+	+	+/-
82	CACUUGGUCACCCAC	130	+	+	+	+/-
83	CACUACACAUUCUCCAC	200	+	+	++	+
84	CACACUCCAUACAC	120	+	+	+	+
85	CACACAUCACCCACAC	170	++	++	++	++

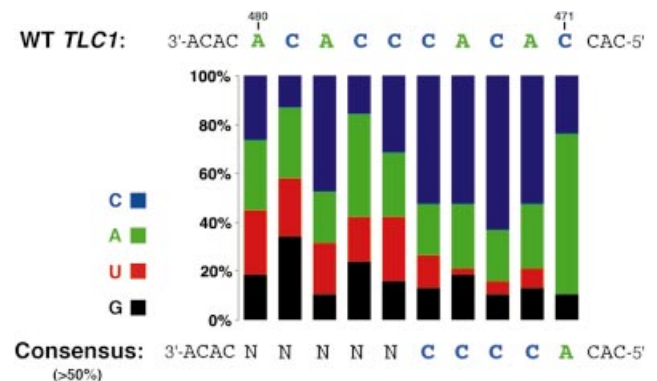


Figure 2. Nucleotide frequencies at the randomized positions in Library₄₈₀₋₄₇₁ of those alleles that complemented a *tlc1* deletion. The color-coded bars show the frequency of the four nucleotides found at the respective position. The consensus indicated at the bottom represents nucleotides that are present in more than half of the complementing template mutations at the indicated positions.

mutant templates, and the relaxed consensus $C_{2-4}(AC)_{1-3}$ is found in 18 of the full-length and two of the non full-length mutant templates (see Table 1). It should be noted, however, that at least the short versions of this consensus (e.g. 3'-CCAC-5') are not sufficient for telomere maintenance; they presumably require additional sequence features within the template (see, for example, the results with Library₄₇₇₋₄₇₃ below).

The template positions 3' of ⁴⁷⁷C make no essential contributions to the sequence-dependent function of telomerase

In wild-type telomerase, >70% of the alignment events take place between positions ⁴⁸⁴A and ⁴⁷⁹C (19). However, no sequence conservation was apparent for positions 480–476 in complementing clones from Library₄₈₀₋₄₇₁. To directly test the sequence requirements in this region, we designed a library in which the nucleotides 3' of ⁴⁷⁷C were mutagenized (Library₄₈₃₋₄₇₈, Fig. 1). The majority of the templates from Library₄₈₃₋₄₇₈ rescued the cells from senescence ($95 \pm 11\%$, average \pm SD, $n = 3$), arguing that this region either does not contribute significantly to sequence-dependent telomerase functions or that substrate annealing 5' of ⁴⁷⁷C can be sufficient for *in vivo* telomere maintenance.

Consecutive rC template nucleotides mediate a sequence-dependent function of TLC1

The consensus template sequence derived from the complementing clones of Library₄₈₀₋₄₇₁ resembles the central ⁴⁷⁷CCCAC⁴⁷³ sequence of wild-type *TLC1* but is found closer to the template 5' boundary. To further test whether the region at the template 5' boundary contributes to the sequence-dependent function, we designed a library in which only the five template positions adjacent to the 5' template boundary were randomized (Library₄₇₂₋₄₆₈, Fig. 1). Only about half of the template sequences from this library could complement the *tlc1* deletion ($40 \pm 12\%$, $n = 3$). Since Library₄₇₂₋₄₆₈ contained fewer complementing template RNAs than Library₄₈₃₋₄₇₈, even though the complexity of Library₄₇₂₋₄₆₈ is lower, we propose that part of the sequence-dependent function resides within positions 472–468 of *TLC1*. We cannot exclude, however, that some templates of Library₄₇₂₋₄₆₈ are non-functional because mutant nucleotides, once incorporated into the telomere, impair the translocation or re-annealing step during the next round of telomere extension.

We next analyzed whether the central ⁴⁷⁷CCCAC⁴⁷³ is important for *TLC1* function. Templates from Library₄₇₇₋₄₇₃ could complement the *tlc1* deletion to roughly the same extent as those from Library₄₇₂₋₄₆₈ ($32 \pm 3\%$, $n = 4$). Thirty-two complementing templates were recovered and sequenced (Table 2). Four templates were recovered twice (4 and 45, 17 and 33, 18 and 47, and 20 and 23), and all of these contained at least a CC dinucleotide in the mutant region. Considering the entire set of the recovered templates, 22 out of the 28 distinct sequences contained a CC dinucleotide or a CCC trinucleotide in the mutagenized region (78% of the complementing templates). This bias is significantly higher than the random frequency of a CC dinucleotide in a 5mer sequence [25% expected: $P = (0.25)^2 \times 4 = 0.25$] and was not found in the library before selection (data not shown).

Table 2. Complementing templates from Library₄₇₇₋₄₇₃

pL5cmp-	Template sequence (3' to 5')
WT	CACACACCCACCACCAC
2	CACACAGUGCCACCACC
3	CACACACCCUCCACCACC
4	CACACACCCACACCACC
5	CACACAUAACCACCACC
6	CACACACAUGCCACCACC
7	CACACAGUUCACCACCACC
8	CACACAGACCCACCACC
9	CACACAACCCGACCACC
12	CACACAUGGCCACCACC
17	CACACACCCUCCACCACC
18	CACACAGUUCACCACCACC
20	CACACAUCUCCACCACC
21	CACACACGCCACCACCACC
23	CACACAUCUCCACCACC
27	CACACACCCGACCACCACC
28	CACACAUGCCACCACCACC
29	CACACACGCCACCACCACC
31	CACACACAGCCACCACCACC
32	CACACAUAACCACCACC
33	CACACACCCUCCACCACC
35	CACACAUCUCCACCACC
38	CACACACUCCACCACCACC
40	CACACAUGCCACCACCACC
45	CACACACCCACACCACC
47	CACACAGUUCACCACCACC
48	CACACAGCCAUACCACC
49	CACACAUCUCCACCACC
50	CACACAGCCUACCACCACC
51	CACACAGCCUACCACCACC
52	CACACAUAACCACCACC
53	CACACACUCCACCACCACC
54	CACACAUAACCACCACC

Furthermore, the consensus ⁴⁷⁷NNNCC⁴⁷³ could be derived. Taken together with the results from Library₄₈₀₋₄₇₁, this indicates that at least two consecutive rC template nucleotides are important for efficient telomerase function. Two separate blocks of consecutive rC nucleotides, as found in the wild-type template sequence, were clearly selected for in the context of Library₄₇₇₋₄₇₃ and therefore appear to enhance telomerase efficiency further.

Few mutant telomerase RNA templates induce a growth arrest in *S.cerevisiae*

The incorporation of mutant sequences at the 3' end of a telomere can interfere with its capping function and can lead to cell death (5,6,12,13,24). Since an inducible expression system for mutant *TLC1* genes using the *GAL₁₋₁₀* promoter did not give satisfactory results (data not shown), we employed a plasmid shuffling technique to recover mutant library plasmids. This approach allowed us to screen for *tlc1* alleles that were recessive to wild-type *TLC1* but lethal when expressed on their own. From a total of 60 000 transformants with Library₄₈₀₋₄₇₁, we obtained only two candidates that reproducibly induced growth arrest in the context of equilibrium-length telomeres (Fig. 3). This corresponds to a frequency of 0.003%, even lower than that determined in the complementation screen with Library₄₈₀₋₄₇₁ (0.07%).

Since the applied selection scheme depends on a recessive phenotype of the *tlc1* template mutation with respect to the wild-type *TLC1* plasmid, we also determined the proportion of Library₄₈₀₋₄₇₁ plasmids that show a dominant phenotype. We observed no reduced viability upon transformation of 12 randomly chosen template library plasmids into *TLC1*

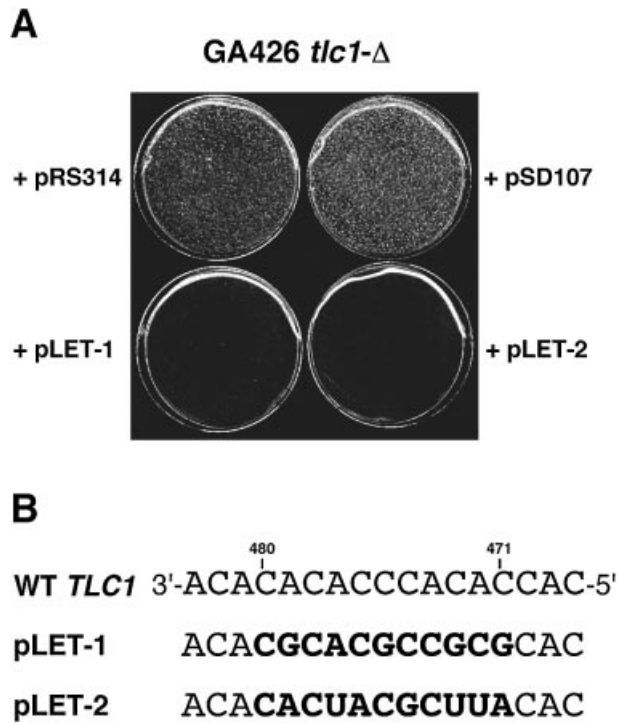


Figure 3. (A) Growth arrest can be induced by template mutant telomerase. *tlc1*-Δ cells were transformed with the indicated plasmids well before the onset of replicative senescence. (B) Two template sequences of death-inducing template RNAs. The tetranucleotide 3'-ACGC-5' is present in both lethal templates, but also in complementing RNA templates from Library₄₇₇₋₄₇₃ (21, identical with 29) and Library₄₈₀₋₄₇₁ (59). It is therefore not solely responsible for the lethal effect.

wild-type cells. Thus, the level of mutant *tlc1* genes in Library₄₈₀₋₄₇₁ with a dominant lethal growth phenotype is likely to be <10% and can therefore not explain the low frequency of plasmids in this library that complemented a *tlc1* deletion.

Template mutant telomerase RNAs are efficiently assembled into telomerase enzymes

We examined whether mutant telomerase RNAs were efficiently assembled into telomerase ribonucleoprotein complexes with an IP-RT-PCR strategy. Since in all template library plasmids an *NcoI* restriction site close to the *TLC1* template region is deleted, the mutant template RNAs can be distinguished from wild-type *TLC1* RNA in a mixed population by digestion with *NcoI*. We transformed a strain carrying a fusion gene of the telomerase catalytic subunit (*EST2*) and protein A with Library₄₈₀₋₄₇₁. The protein A-tagged telomerase was immunoprecipitated from protein extracts with IgG-coated beads (20,25) and the associated RNA was reverse transcribed. A fragment around the *TLC1* template region was amplified by PCR, digested with *NcoI* and analyzed by gel electrophoresis (Fig. 4A). The wild-type *TLC1* RNA present in this strain served as a positive control for the efficiency of the IP. We found that eight randomly chosen template mutants were incorporated into telomerase enzymes as efficiently as wild-type *TLC1* (Fig. 4B). Thus, the assembly of telomerase does not show sequence specificity

with regard to the template region. This is consistent with the finding that the *TLC1* template region is dispensable for assembly with the catalytic subunit, Est2p (26).

Enforced interaction of telomerase with the telomeres does not increase the frequency of functional template RNAs

Telomerase is recruited to or activated at telomeres through the interaction of Est1p with Cdc13p (27-31). Fusions of the open reading frames of telomerase components and *CDC13* or its DNA-binding domain (*CDC13*_{DBD}) force the interaction of telomerase with the telomere and lead to vigorous telomere elongation (27,32,33). We included two such fusion proteins, Cdc13-Est2p (27) and Cdc13_{DBD}-Est3p (32), in our screen for growth arrest. If the mutant telomerases were inactive due to an access defect, the *CDC13* fusion proteins should have alleviated this problem.

In the presence of either Cdc13 fusion protein, the frequency of colonies that did not grow after counterselection of the wild-type *TLC1* plasmid varied only slightly between the empty vector ($3.1 \pm 0.75\%$, $n = 2$), the wild-type plasmid ($2.3 \pm 0.3\%$, $n = 2$) and the Library₄₈₀₋₄₇₁ ($5.3 \pm 1.6\%$, $n = 2$). Twenty-four colonies without an apparent growth defect after counterselection of the wild-type *TLC1* plasmid were restreaked successively, and all showed senescence at the same time as control clones with the empty vector (data not shown). In addition, the overall transformation efficiency of the library was not reduced by the introduction of the fusion proteins, indicating that the frequency of dominant lethal template mutations also did not increase.

The presence of the Cdc13-telomerase fusion proteins therefore does not rescue the *in vivo* incorporation defect seen with the majority of the mutant templates in Library₄₈₀₋₄₇₁. Telomere elongation in the presence of the Cdc13-telomerase fusion proteins did occur in the presence of a wild-type *TLC1* gene (Fig. 5, compare lane 1 with lanes 2-10). However, the elongated telomeres became shortened in cells with the empty vector or library plasmids after the wild-type *TLC1* plasmid had been shuffled out (Fig. 5, lanes 14-19).

Most templates in Library₄₈₀₋₄₇₁ do not lead to incorporation of mutant sequences into the telomeres

The frequency at which candidate templates were obtained in either of the two screens was <0.1% of Library₄₈₀₋₄₇₁. Since the mutant *tlc1* RNAs were efficiently assembled into telomerase enzymes and most likely not limited in their access to the telomeres, we examined whether the telomeres had acquired mutant sequences. We employed telomere-PCR (21) to compare the telomere length of cells that contained either a wild-type *TLC1* gene, an empty vector or a plasmid from Library₄₈₀₋₄₇₁. While the presence of wild-type *TLC1* allowed normal telomere length maintenance (266 ± 18 nt, $n = 20$), cells that had received an empty vector lacked telomerase activity and consequently had significantly shorter telomeres (179 ± 34 nt, $n = 20$, shorter than wild-type *TLC1* $P < 0.001$, *t*-test) 25 generations after a plasmid containing wild-type *TLC1* had been shuffled out. The telomere length of cells that contained a plasmid from Library₄₈₀₋₄₇₁ showed the same extent of telomere shortening (188 ± 34 nt, $n = 20$, shorter than wild-type *TLC1* $P < 0.001$, and shorter than empty vector $P > 0.4$, *t*-test), and these mutant yeast cells senesced

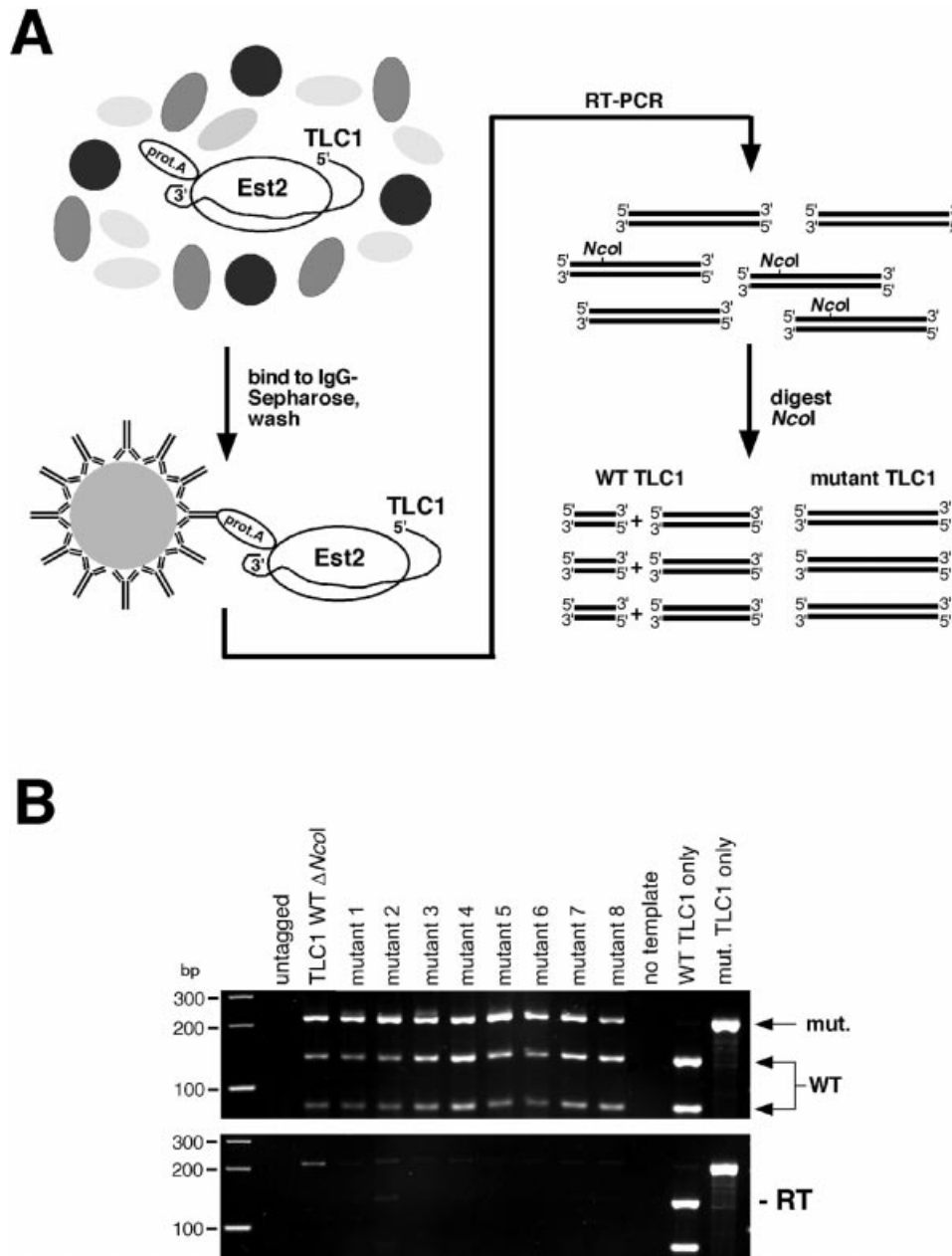


Figure 4. Template mutant RNAs are associated with Est2p. (A) Outline of the IP-RT-PCR experiment to assess the association of template mutant telomerase RNAs with the telomerase catalytic subunit, Est2p. All yeast strains contained a wild-type *TLC1* gene, which serves as internal control for IP efficiency and as competitor for the assembly of the template mutant RNAs with Est2p *in vivo*. (B) All eight randomly chosen template mutants (mutants 1–8) from Library_{480–471} are efficiently assembled into telomerase enzymes. Upper panel, PCR products can be obtained for both the endogenous wild-type *TLC1* RNA (cut with *NcoI*) and the template mutant *TLC1* RNAs (larger, uncut fragment). *TLC1* WT $\Delta NcoI$ refers to a control mutation where the *NcoI* site was abolished but the template sequence was left unchanged. Lower panel, PCR amplification of the immunopurified RNA without prior RT treatment to reveal products due to DNA contamination.

upon further re-streaking. To rule out the possibility of very low levels of mutant sequence incorporation, 12 telomeres from four different template mutants were cloned but no mutant sequences were recovered.

Non-functional template mutations affect the nucleotide addition processivity of telomerase

We compared the *in vitro* telomerase activities of complementing and non-complementing template mutants obtained from Library_{477–473}. The candidates were taken from this

library because the same DNA oligonucleotide substrate, d(TG)₇, can be used for all mutants. It presumably anneals within the sequence ⁴⁸⁴ACACACA⁴⁷⁶ which is present in all mutant templates [note that in the context of a wild-type template sequence, the nucleotides ⁴⁷⁹CAC⁴⁷⁷ and ⁴⁷³CAC⁴⁷¹ are excluded for initial substrate annealing *in vivo* (19)]. Telomerase was prepared from cells carrying either the wild-type *TLC1* gene, an empty vector, one of three non-complementing telomerase RNA template mutants (chosen from a random collection of individual mutant templates from

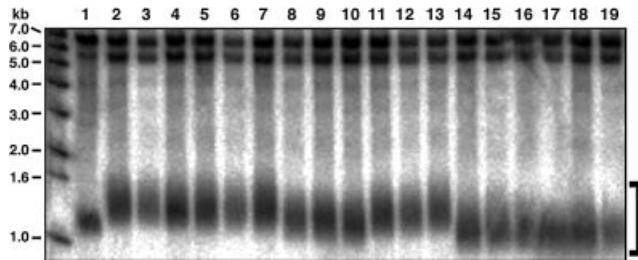


Figure 5. Telomere length analysis by Southern blotting of cells expressing a Cdc13_{DBD}-Est3 fusion protein and various template mutant telomerase RNAs. Except for the wild-type strain, three independent colonies were analyzed for each genotype. Genomic DNA was prepared 50–75 generations after introduction of the Cdc13_{DBD}-Est3 fusion protein. The library candidates were chosen at random. Lane 1, GA426 WT; lanes 2–4, YKF20 + pVL1292 + pTLC1-URA3 + pSD107; lanes 5–7, YKF20 + pVL1292 + pTLC1-URA3 + pRS314; lanes 8–10, YKF20 + pVL1292 + pTLC1-URA3 + candidates from Library_{480–471}; lanes 11–13, YKF20 + pVL1292 + pSD107, pTLC1-URA3 shuffled out; lanes 14–16, YKF20 + pVL1292 + pRS314, pTLC1-URA3 shuffled out; lanes 17–19, YKF20 + pVL1292 + candidates from Library_{480–471}, pTLC1-URA3 shuffled out. Terminal restriction fragments of the Y'-telomeres are indicated by the bracket on the right.

Library_{477–473}) or one of three complementing telomerase RNA template mutants. Strikingly, all three non-complementing template mutants showed a reduced activity compared with wild-type and complementing mutant telomerases (Fig. 6).

The low activity levels detected might have been caused by the changed template sequence, resulting in a lower incorporation rate of the labeled nucleotide [compare, for example, complementing mutant 2 in Figure 6 with labeled dTTP (second panel) and labeled dGTP (third panel)]. We tested this hypothesis by including labeled dATP in the reaction. The non-complementing mutant templates tested in our assay all contain an rU nucleotide at the first mutant position, which should direct the incorporation of the labeled dATP. No increased activity for the non-complementing mutants was detected in these reactions (Fig. 7A), confirming an impaired enzymatic activity.

Despite their reduced activity, the non-complementing template mutant telomerases could add a single nucleotide to the substrate DNA oligonucleotide (TG)₇ (e.g. see Fig. 6, second panel) and up to 3 nt to the substrate oligonucleotide (GT)₇ (Fig. 6, bottom panel). This activity was RNase sensitive (Fig. 7B), absent in extracts from cells that had received an empty vector instead of a TLC1 gene (e.g. Fig. 6, second panel, first lane) and weak when labeled dGTP was used in the reaction with the substrate (TG)₇ (Fig. 6, third panel). We therefore conclude that it represents bona fide template-directed telomerase activity, strongly suggesting that the processivity is perturbed in the mutant enzymes. This may result either from an inefficient incorporation of dATP and dCTP by telomerase or from an inability of the mutant enzymes to perform structural transitions necessary to advance to the next template position. It is unlikely that the templating nucleotide ⁴⁷⁷U is solely responsible for the enzymatic defect, as many complementing mutants from Library_{477–473} also contain the mutation ⁴⁷⁷U (Table 2). Furthermore, we have shown previously that a ⁴⁶⁹A→U mutation leads to the incorporation of dA into telomeres *in vivo* (19), arguing that dATP can be a substrate for yeast telomerase. The low frequency of functional template RNAs

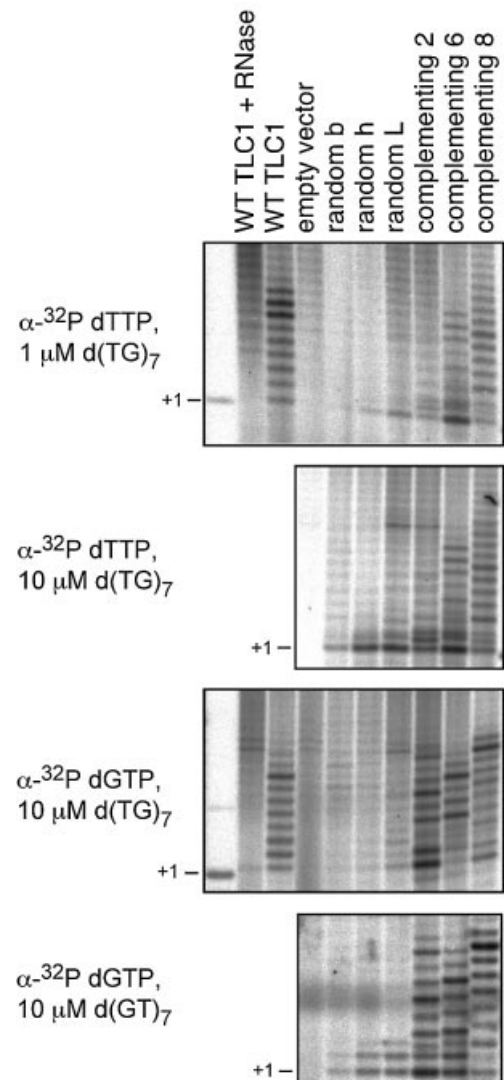


Figure 6. *In vitro* telomerase activity assays. The DNA oligonucleotide substrate and the labeled dNTP are indicated on the left. TLC1 template mutants were selected from Library_{477–473}. The reactions contained all unlabeled dNTP substrates at 50 μM and labeled dNTP substrates at 5 μM. The template sequences were (mutant stretches underlined): random b ⁴⁸⁴ACA-CACAUGGAGACCAC⁴⁶⁸, random h ⁴⁸⁴CACACAUAGUGACCAC⁴⁶⁸, random L ⁴⁸⁴CACACAUCUAGACCAC⁴⁶⁸, complementing 2 ⁴⁸⁴CACACA-GUGCCACCAC⁴⁶⁸, complementing 6 ⁴⁸⁴CACACACAUGCACCAC⁴⁶⁸ and complementing 8 ⁴⁸⁴CACACAGACCCACCAC⁴⁶⁸.

contained in Library_{477–473} and, most probably, Library_{480–471} therefore appears to be due to an impact of mutant template sequences on telomerase nucleotide addition processivity. While this reduced processivity is the most striking phenotype of the template mutant telomerases, we cannot exclude additional effects on, for example, the DNA substrate affinity or the fidelity of the telomerase enzyme.

DISCUSSION

Functional requirements on the telomerase RNA template sequence

Our template library screens reveal for the first time that only a minor fraction of all possible template sequences will

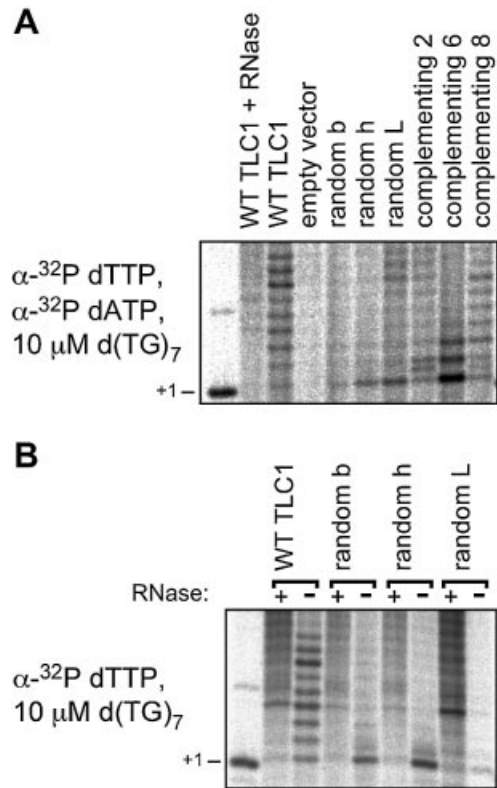


Figure 7. *In vitro* telomerase activity assays. (A) Control reactions containing both labeled dTTP and labeled dATP. No increased activity could be detected for the non-complementing telomerase RNA template mutations (random b, random h and random L). (B) The +1 band obtained in the reactions with the non-complementing telomerase RNA template mutations is RNase sensitive.

reconstitute active telomerase in *S.cerevisiae*. Functional templates from Library_{480–471} often contained the sequence 3'-C_{2–4}(AC)_{1–3}-5' positioned at or near the template 5' boundary. This motif resembles the central ⁴⁷⁷CCCAC⁴⁷³ and the 5' template boundary ⁴⁷¹CCAC⁴⁶⁸ of wild-type *TLC1*, indicating that these sequences play a role in telomerase biochemistry that goes beyond their function as a passive template for nucleotide addition. Analysis of template mutant telomerases *in vitro* revealed that non-complementing template mutations lead to reduced nucleotide addition processivity. Similar results were reported previously for *T.thermophila* telomerase based on a series of site-specific RNA template mutants (14–16). The present study not only extends this notion to budding yeast, but also explores 60 000 different template sequences in parallel.

In wild-type *TLC1*, the nucleotides ⁴⁷⁹CAC⁴⁷⁷ and ⁴⁷³CAC⁴⁷¹ are not available for telomere alignment but become available for base pairing during reverse transcription. In addition, the central ⁴⁷⁷CCC⁴⁷⁵ trinucleotide is reverse transcribed in a processive manner, while product dissociation can occur 3' or 5' of this motif (19). Thus, the biochemical properties of telomerase vary with the template position that is being reverse transcribed. Also, defined changes in the sequence of the RNA template could influence telomerase activity. For example, the mutation ⁴⁷⁶CCA⁴⁷⁴→GUG in *S.cerevisiae* telomerase RNA gave rise to an inactive enzyme

(18) when it was the only telomerase RNA species in the cell. However, telomerase activity was restored even for the mutant template in the context of wild-type/mutant enzyme heteromultimers. It is unclear at this point which enzymatic defect is conveyed by this mutation. Whatever the rules governing the properties of yeast telomerase may be, the reduced *in vitro* processivity of the three template mutant telomerases tested in our study indicates that the threading of subsequent template nucleotides through the active site is very sensitive to changes in the RNA template sequence. A previous study has shown that a mutant yeast telomerase RNA specifying human telomeric repeats is functional *in vivo* and leads to the incorporation of human telomere repeats onto yeast chromosome ends (34). This mutation corresponds roughly to our derived consensus as it contains several CCC trinucleotides. However, since the putative total length of the template region is longer, this mutant template sequence cannot be superimposed without prior assumptions onto the results obtained in our study. Several point mutations in the budding yeast telomerase catalytic subunit (*Est2p*) resulted in reduced nucleotide addition processivity *in vitro* (35,36). It is not clear whether these *est2* mutations affect the same mechanism as our RNA template mutations, especially since the deficiency conferred by RNA template sequence changes is far more pronounced.

Our screen also demonstrates that a stretch of successive rC template nucleotides is not absolutely required for telomere maintenance (see, for example, template 65 in Table 1) and that the sequence ⁴⁷²CCAC⁴⁶⁸ is not sufficient for telomerase activity *in vivo* (complementation <100% with Library_{477–473}). The sequence requirements on the budding yeast RNA template therefore must be more complex than the minimal consensus found in our study. It is noteworthy that in the context of the very long telomerase RNA template regions of certain yeasts, the telomerase RNA templates do not need to be particularly C/A rich (4,37).

The active templates identified in our screen may have been subject to a further selection for telomerases that synthesize DNA with binding sites for essential telomere-binding proteins such as Rap1p, Cdc13p and Est1p. While Cdc13p provides essential end-protecting functions and recruits or activates telomerase via its interaction with Est1p (28,29,31,38–40), the binding of Rap1p to the telomeres negatively regulates extension (41–43). Thus, a fully functional telomerase that does not incorporate Rap1p-binding sites into the telomere is predicted to lead to strong telomere elongation. Mutant telomerase enzymes with this phenotype can be obtained through single nucleotide substitutions in the telomerase RNA template region (4,7,44), leading to rapid telomere elongation by >2 kb within 50 generations (7,44). In contrast, almost all of the complementing template mutations identified in our screen of Library_{480–471} resulted in shortened telomeres. Since it is unlikely that all the corresponding mutant telomere sequences result in increased Rap1p binding, we propose that the mutant telomerases do not obtain the full activity of the wild-type enzyme. This hypothesis is corroborated by the reduced *in vitro* nucleotide addition processivity of non-complementing template mutant telomerases. On the other hand, incorporated mutant telomere sequences that lead to reduced binding of Cdc13p (a complete lack of Cdc13p binding should be lethal) may decrease the

recruitment or activation of telomerase. Even in the context of a Cdc13_{DBD}-telomerase fusion protein, this could have prevented the generation of long stretches of mutant telomeric DNA and thus may have limited the number of complementing RNA template sequences recovered in our screens.

Has the need for C/A-rich template RNAs contributed to the conservation of the telomeric repeat sequences?

The relatively strong conservation of the telomeric repeat sequence during evolution is an unusual feature for non-coding DNA. The propensity of single-stranded telomeric DNA from most species to form stable secondary structures based on G-G pairing has been proposed to play a protective role at the chromosome end. Loss of the telomeric 3' single-stranded extensions correlates with loss of end protection (45), and experimentally induced chromosome end-to-end fusions occurred preferentially at telomeres replicated by the leading strand machinery (46), which leaves blunt ends after replication. This putative protective function of the telomere sequence could certainly explain its conservation.

On the other hand, the template sequence dependence of telomerase activity described here may also limit the divergence of telomeric sequences during evolution. Consistent with this hypothesis, the telomeres of *Drosophila melanogaster*, which are maintained by retrotransposition rather than telomerase, are not G/T rich [reviewed by Pardue *et al.* (47) and Louis (48)]. Their chromosome ends nonetheless are specifically recognized and protected since mutations in a telomere-binding protein lead to chromosome end-to-end fusions (49). This indicates that telomere capping can be achieved without the help of G-rich DNA secondary structures and that telomere sequences can, in principle, deviate from the T/G-rich consensus. Our template library screen has revealed that extensive changes of the budding yeast telomerase RNA template sequence most probably result in non-functional telomerase enzymes. If this specialization of the TERT enzyme for a C/A-rich RNA template is conserved in other telomerases, it may give an alternative explanation for the conservation of the telomere sequence.

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