Winter 1-22-2003

Yeast Telomerase is Specialized for C/A-rich RNA Templates

Klaus Förstemann  
*Swiss Institute for Experimental Cancer Research*

Arthur J. Zaug  
*University of Colorado Boulder*

Thomas R. Cech  
*University of Colorado Boulder, thomas.cech@colorado.edu*

Joachim Lingner  
*Swiss Institute for Experimental Cancer Research*

Follow this and additional works at: [https://scholar.colorado.edu/chem_facpapers](https://scholar.colorado.edu/chem_facpapers)

Part of the [Biochemistry Commons](https://scholar.colorado.edu/chem_facpapers), and the [Molecular Biology Commons](https://scholar.colorado.edu/chem_facpapers)

Recommended Citation  
[https://scholar.colorado.edu/chem_facpapers/9](https://scholar.colorado.edu/chem_facpapers/9)

This Article is brought to you for free and open access by Chemistry & Biochemistry at CU Scholar. It has been accepted for inclusion in Chemistry & Biochemistry Faculty Contributions by an authorized administrator of CU Scholar. For more information, please contact cuscholaradmin@colorado.edu.
Yeast telomerase is specialized for C/A-rich RNA templates

Klaus Förstemann, Arthur J. Zaug¹, Thomas R. Cech¹ and Joachim Lingner*  

Swiss Institute for Experimental Cancer Research (ISREC), Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland and ¹Department of Chemistry and Biochemistry, Howard Hughes Medical Institute, University of Colorado at Boulder, Boulder, CO 80309-0215, USA

Received December 5, 2002; Revised and Accepted January 22, 2003

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 template sequences 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 telomerasers (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

*To whom correspondence should be addressed. Tel: +41 21 6925912; Fax: +41 21 6526933; Email: joachim.lingner@isrec.unil.ch
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 477CCCAC477 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-GAGAAGGCTACATACACCCACACAATTGTTA-CAG-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 (Library483–471, 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 can-1 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 growth without TLC1 RNA (empty vector), the same amount of protein as for the wild-type preparation was employed.

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...
central 10 nt of the wild-type \textit{TLC1} template region (Library\textsubscript{480-471}, 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 \textit{TLC1} gene, we transformed YKF19 (\textit{tlc1-D rad52-D}) with Library\textsubscript{480-471} 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 \textit{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 \textit{TLC1} template region and were not taken into account in deriving the consensus sequence. Most telomerase RNA template mutations that complemented the \textit{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: \(P > 99.9, \chi^2\) 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'-'CCCACAC-5', reminiscent of the central portion in the wild-type \textit{TLC1} template sequence, can fulfill the sequence-specific requirements for \textit{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 Library\textsubscript{480-471}

<table>
<thead>
<tr>
<th>pLib-</th>
<th>template sequence (3' to 5')</th>
<th>telomere length (nt T\textsubscript{G,Gt})</th>
<th>23°C</th>
<th>25°C</th>
<th>30°C</th>
<th>36°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>CACACACCCACACCCACAC</td>
<td>280</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>7</td>
<td>CACGUTDRCCACAC</td>
<td>130</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>9</td>
<td>CACGUGACCGCAC</td>
<td>170</td>
<td>++</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>11</td>
<td>CACGUGACCGCAC</td>
<td>130</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>14</td>
<td>CACGCCGACCGCAC</td>
<td>150</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>CACCTAACCACAC</td>
<td>230</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>22</td>
<td>CACGUGACCGCAC</td>
<td>140</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>CACGUGACCGCAC</td>
<td>200</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>24</td>
<td>CACGUGACCGCAC</td>
<td>140</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>28</td>
<td>CACGUGACCGCAC</td>
<td>150</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
<td>CACGUGACCGCAC</td>
<td>160</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
<td>CACCTAACCACAC</td>
<td>310</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>47</td>
<td>CACGUGACCGCAC</td>
<td>170</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>48</td>
<td>CACGUGACCGCAC</td>
<td>140</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>50</td>
<td>CACGUGACCGCAC</td>
<td>150</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>52</td>
<td>CACGUGACCGCAC</td>
<td>130</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>54</td>
<td>CACGUGACCGCAC</td>
<td>150</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>55</td>
<td>CACGUGACCGCAC</td>
<td>270</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>56</td>
<td>CACGUGACCGCAC</td>
<td>180</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>59</td>
<td>CACGUGACCGCAC</td>
<td>160</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>CACGUGACCGCAC</td>
<td>150</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>CACGUGACCGCAC</td>
<td>150</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>62</td>
<td>CACGUGACCGCAC</td>
<td>n.d.</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>63</td>
<td>CACGUGACCGCAC</td>
<td>230</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>CACGUGACCGCAC</td>
<td>180</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>66</td>
<td>CACGUGACCGCAC</td>
<td>n.d.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>CACGUGACCGCAC</td>
<td>210</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>CACGUGACCGCAC</td>
<td>190</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>CACGUGACCGCAC</td>
<td>180</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>CACGUGACCGCAC</td>
<td>n.d.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>CACGUGACCGCAC</td>
<td>180</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>CACGUGACCGCAC</td>
<td>180</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>CACGUGACCGCAC</td>
<td>180</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>CACGUGACCGCAC</td>
<td>160</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>CACGUGACCGCAC</td>
<td>160</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>79</td>
<td>CACGUGACCGCAC</td>
<td>160</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>CACGUGACCGCAC</td>
<td>150</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>81</td>
<td>CACGUGACCGCAC</td>
<td>n.d.</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>82</td>
<td>CACGUGACCGCAC</td>
<td>130</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>CACGUGACCGCAC</td>
<td>200</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>CACGUGACCGCAC</td>
<td>120</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>CACGUGACCGCAC</td>
<td>170</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** Schematic representation of the different \textit{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.

**Figure 2.** Nucleotide frequencies at the randomized positions in Library\textsubscript{480-471} of those alleles that complemented a \textit{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.
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 477C (19). However, no sequence conservation was apparent for positions 480–476 in complementing clones from Library 480–471. To directly test the sequence requirements in this region, we designed a library in which the nucleotides 3' of 477C were mutagenized (Library 483–478, Fig. 1). The majority of the templates from Library 483–478 rescued the cells from senescence (95 ± 11%, average ± 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 Library 480–471 resembles the central 477CCAC473 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 (Library 472–468, Fig. 1). Only about half of the template sequences from this library could complement the tlc1 deletion (40 ± 12%, n = 3). Since Library 472–468 contained fewer complementing template RNAs than Library 483–478, even though the complexity of Library 472–468 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 Library 472–468 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 Library 477–473 could complement the tlc1 deletion to roughly the same extent as those from Library 472–468 (32 ± 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 × 4 = 0.25] and was not found in the library before selection (data not shown).

Furthermore, the consensus 477NNNCC473 could be derived. Taken together with the results from Library 480–471, 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 Library 477–473, 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 promotor 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 Library 480–471, 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 0.063%, even lower than that determined in the complementation screen with Library 480–471 (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 Library 480–471 plasmids that show a dominant phenotype. We observed no reduced viability upon transformation of 12 randomly chosen template library plasmids into TLC1
The assembly of telomerase does not show sequence specificity for the efficiency of the IP. We found that eight randomly TLC1 RNA present in this strain served as a positive control analyzed by gel electrophoresis (Fig. 4A). The wild-type template region was amplified by PCR, digested with NcoI and the associated tagged telomerase was immunoprecipitated from protein extracts with IgG-coated beads (20,25) and the associated fusion proteins did occur in the presence of a wild-type TLC1-ACGC-5’ template region close to the NcoI restriction site (21, identical with 29) and Library477–473 (59). It is therefore not solely responsible for the lethal effect.

**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 (CDC13DBD) 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 Cdc13DBD–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 ± 0.75%, n = 2), the wild-type plasmid (2.3 ± 0.3%, n = 2) and the Library480–471 (5.3 ± 1.6%, n = 2). Twenty-four colonies without an apparent growth defect after counterselection of the wild-type TLC1 plasmid were re-screened 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
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 480–471. 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 5'ACACACA476 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 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 ΔNcoI 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.
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 \(^{477}U\) is solely responsible for the enzymatic defect, as many complementing mutants from Library\(_{477-473}\) also contain the mutation \(^{477}U\) (Table 2). Furthermore, we have shown previously that a \(^{469}A\rightarrow U\) 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 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
reconstitute active telomerase in *S. cerevisiae*. Functional templates from Library$_{480-471}$ often contained the sequence $3'$-$C_2$-$d(AC)_{1-3}$-$5'$ positioned at or near the template $5'$ boundary. This motif resembles the central $47'$-$CCCAC$$_{473}$ and the $5'$ template boundary $47'$-$CCAC$$_{468}$ 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 mutant mutants (14–16). The present study not only extends this notion to budding yeast, but also explores 60 000 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 $472'$-$CCAC$$_{468}$ 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 Cdc13DBD-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 telomerasers, it may give an alternative explanation for the conservation of the telomere sequence.

ACKNOWLEDGEMENTS

The authors thank Dr V. Lundblad for plasmids encoding the Cdc13-Est2 (pVL1107) and Cdc13DBD-Est3 (pVL1292) fusion proteins. This work was supported by a Human Frontier Science Program grant (laboratories of J.L. and T.R.C.), the Swiss National Science Foundation (J.L.) and the National Center of Competence in Research ‘Frontiers in Genetics’ program (J.L.).

REFERENCES


