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Telomerase limits the extent of base pairing between template RNA and telomeric DNA

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Telomerase is the ribonucleoprotein reverse transcriptase that adds telomeric DNA repeats to the ends of chromosomes. This involves annealing of the telomerase RNA template to the 3′ end of the chromosome, reverse transcription of the RNA template by the telomerase reverse transcriptase polypeptide and translocation. Here, we overexpress and partially purify the catalytically active yeast telomerase core in its natural host and probe telomerase RNA base methylation accessibility with dimethyl sulphate in the presence and absence of a DNA substrate and after substrate elongation. The length of the RNA–DNA hybrid is kept constant at seven base pairs after primer binding and elongation. Thus, new base-pair formation at the 3′ end of the substrate during elongation coincides with disruption of base-pair interactions at the other side of the template. Presumably, this circumvents the generation of an exceedingly high energy barrier for translocation and dissociation. Our analysis also corroborates recently proposed yeast telomerase RNA secondary structure models.

Keywords: telomerase reaction cycle; telomerase RNA structure; dimethyl sulphate; reverse transcriptase

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INTRODUCTION

The telomerase catalytic core is provided by the telomerase reverse transcriptase (TERT) polypeptide, which reverse transcribes a short stretch of the telomerase RNA moiety (TER) to extend the chromosome 3′ ends. TERT is thought to function similarly to reverse transcriptases from retroviruses and retroelements because they share highly conserved sequence motifs (Kelleher *et al*, 2002). However, reverse transcription by telomerase involves re-setting the relative positions of the RNA template and telomeric substrate in the active site after the addition of each telomeric repeat. Thus, in addition to the RT motifs, TERT contains one or two amino-terminal RNA-binding domains that bind TER outside the template and that may enable stable association with telomerase RNA while allowing the template to move through the active site (Bryan *et al*, 2000). Other telomerase-associated proteins have a role in telomerase assembly and its regulation at chromosome ends *in vivo*, but they are not required for enzymatic activity *in vitro* (Evans & Lundblad, 2000).

Single-stranded telomeric DNA is bound by telomerase through base pairing with the RNA template and through protein–DNA interactions at the so-called anchor site. Measurement of the dissociation rates of different telomeric oligonucleotide substrates with telomerase from *Euplotes aediculatus* showed that substrate affinity was largely independent of the extent of base complementarity between primer and template (Hammond & Cech, 1998). This suggested that either the protein–DNA interaction could compensate for different binding energies stemming from a variable number of base pairs or that telomerase maintains a more or less constant RNA–DNA hybrid length during the reaction cycle. In a study with *Tetrahymena* telomerase with mutant telomerase RNA templates and various DNA substrates, it was concluded that the requirement for potential pairing between primer 3′ end and template increases as catalysis moves along the template (Wang *et al*, 1998).

The comparison of the telomerase RNA sequences from different phyla demonstrates that this RNA diverged quickly in evolution. The sizes range from 150–200 nucleotides (nt) in ciliates to about 450 nt in vertebrates and about 1,200 nt in *Saccharomyces*. The telomerase RNA sequences can be aligned with confidence only among closely related organisms, and secondary structure models were derived for the different groups by phylogenetic comparison (Romero & Blackburn, 1991; Lingner *et al*, 1994; Chen *et al*, 2000; Dandjinou *et al*, 2004; Zappulla & Cech, 2004). In addition, chemical probes and structure-specific RNases have been instrumental in evaluating the secondary structure models (Bhattacharyya & Blackburn, 1994; Zaug & Cech, 1995; Sperger & Cech, 2001; Antal *et al*, 2002; Dandjinou *et al*, 2004). Despite the divergence in sequence and size, the template of all telomerases is single stranded, and all telomerase

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RNAs have been proposed to contain a pseudoknot domain. The potential of the telomerase RNA to form a pseudoknot structure is well supported through nucleotide covariation in ciliates (ten Dam *et al*, 1991; Lingner *et al*, 1994) and vertebrates (Chen *et al*, 2000), and alternative pseudoknot structures have also been proposed for telomerase RNAs from *Saccharomyces* (Chappell & Lundblad, 2004; Dandjinou *et al*, 2004; Lin *et al*, 2004) and *Kluyveromyces* (Tzfati *et al*, 2003).

Here, we overexpress the TERT (Est2) and TER (Tlc1) subunits from yeast and probe the telomerase RNA structure with dimethyl sulphate (DMS) in the absence and presence of DNA substrate primers and after substrate elongation. Our results indicate that telomerase maintains a constant number of base pairs between template RNA and telomeric DNA substrate. In addition, our results corroborate and refine the telomerase RNA secondary structure models that were recently proposed for *Saccharomyces cerevisiae*.

RESULTS AND DISCUSSION

To obtain sufficient amounts of telomerase for structural probing, we co-overexpressed the telomerase reverse transcriptase Est2 and the RNA moiety Tlc1 with the Gal1 promoter in *S. cerevisiae*. These two components form the catalytic core of telomerase, and their overexpression leads to increased *in vitro* telomerase activity without strongly affecting telomere length (Teixeira *et al*, 2002). Overexpressed telomerase was enriched through purification over a DEAE column (see Methods). The telomerase RNA in this fraction was probed for accessibility by the methylating agent DMS in the template region (Fig 1) and other parts of the RNA (Fig 2; supplementary Fig 1 online). Our analysis covers the essential core region described previously (Livengood *et al*, 2002). DMS methylation at position N-1 of A and N-3 of C in RNA occurs readily in single-stranded RNA, but the bases become protected after base-pair formation or protein binding. The modified positions are detected as stop sites during primer extension by reverse transcriptase.

The template region of telomerase RNA

Telomerase from *S. cerevisiae* has the peculiarity of not dissociating from bound telomeric DNA oligonucleotides *in vitro* (Prescott & Blackburn, 1997). We took advantage of this stability to 'lock' the enzyme in different states of the reaction cycle. We found that in the absence of DNA substrate, the entire template region of TLC1 was accessible to modification by DMS (Fig 1A), whereas in the absence of DMS, no stops of the reverse transcription reaction were observed in this region (Fig 1A, right panel). The accessibility to modification by DMS is consistent with the ability of the template to bind chromosome end substrates and is in accordance with the results obtained for *Tetrahymena* telomerase (Zaug & Cech, 1995) and recently proposed models for *S. cerevisiae* telomerase RNA (Dandjinou *et al*, 2004; Zappulla & Cech, 2004). Starting at three nucleotides 5′ of the template, seven bases in a row are completely protected. Strikingly, these bases have been proposed to form one strand of a stem–loop structure involved in defining the 5′ boundary of the template (Seto *et al*, 2003). On addition of a telomerase substrate before modification with DMS, the central portion of the template became protected as expected from the base complementarity of DNA substrate and RNA (Fig 1B). However, the protected region was only 7 bp long, starting at the DNA 3′ end. Closer to the 3′ boundary of the RNA template, the remaining three bases were readily modified by DMS even though they were complementary to the substrate. Thus, although these bases were accessible to DMS, they did not engage in base pairing. We also noted that the presence of substrate primer increased pausing or abortion of the reverse transcription reaction at G446 and G449. However, as G is not protected from DMS modification due to base pairing, the interpretation of this result is not straightforward.

To determine the protection pattern after substrate elongation, dNTPs were added to the telomerase–telomeric primer complex before DMS modification. The shift in the DMS protection pattern indicates that most of the bound substrates became elongated by five nucleotides (Fig 1C), as expected from analysis of product length obtained under similar reaction conditions (Prescott & Blackburn, 1997; Forstemann & Lingner, 2001; Teixeira *et al*, 2002; supplementary Fig 2 online). This result also indicates that most of the telomerase RNA molecules were assembled with Est2 in a catalytically active enzyme. Again, only seven bases of the telomerase RNA engaged in base pairing with the extended substrate, starting at the new DNA 3′ end. Thus, base-pair formation at the 3′ end of the substrate during elongation coincided with base-pair disruption at the 5′ end. The measured K_m of the same substrate was about 0.8 μM (supplementary Fig 2 online), which is consistent with the free energy calculated for the formation of a 7–8 bp RNA–DNA hybrid (see legend to supplementary Fig 2 online for details). This assumes that the interaction of the telomerase protein with the DNA substrate does not contribute substantially to the binding energy. The short substrate oligonucleotides used in our study are too short to interact with the presumed anchor site in *S. cerevisiae* telomerase (Lue & Peng, 1998).

The putative pseudoknot domain

Two alternative pseudoknot structures have been proposed for the telomerase RNA from *S. cerevisiae* (Dandjinou *et al*, 2004; Lin *et al*, 2004; Fig 2B). Significantly, both pseudoknot structures include one stem, corresponding to CS3/CS4 in the lower pseudoknot structure in Fig 2B or to the lower part of stem VI in the upper structure, the base-pair potential of which has been demonstrated to be important for binding the yeast TERT Est2 (Chappell & Lundblad, 2004; Lin *et al*, 2004). Our DMS modification analysis (Fig 2A) is consistent with the existence of the CS3/CS4 stem or the extended bulged stem VI, as all adenines and cytosines in these stems are protected from modification. Also, the DMS protection is consistent with the existence of stem ϕK1 of the upper pseudoknot, whereas A758–762 in the extended H4 helix were accessible to DMS, indicating that stem H4 may be shorter than indicated, or that it may not be stably formed. Three adenines in stem V (A707, A709 and A710) of the upper structure were also strongly modified by DMS, indicating that this stem does not stably form in the Tlc1–Est2 catalytic core complex analysed here.

The Est1 binding bulged stem

The DMS modification data are in excellent agreement with the proposed secondary structures for stems IIa, III and IVa–c. Stem IVc corresponds to the previously described Est1 binding bulged stem (Seto *et al*, 2002). C651 in the bulge of IVc (Fig 2B, asterisk)

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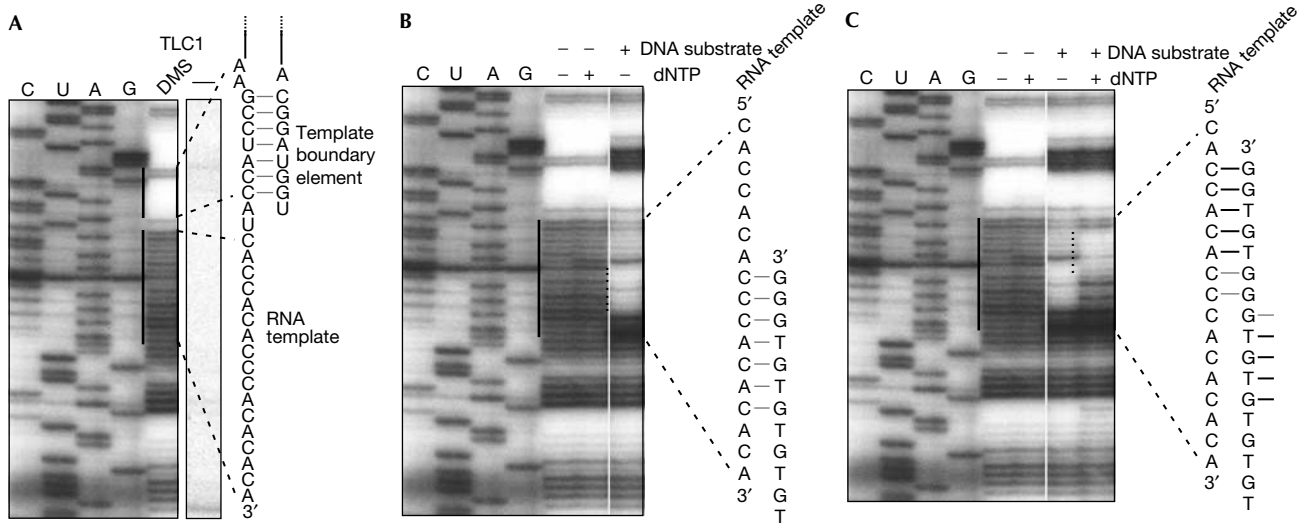

Fig 1 | DMS accessibility of the telomerase RNA template region analysed by primer extension with reverse transcriptase. (**A**) Lanes C, U, A and G denote dideoxynucleotide sequencing reactions using *TLC1*-containing plasmid DNA as a template. DMS denotes the lane in which enriched telomerase fraction was treated with DMS, RNA was extracted and TLC1 RNA was reverse transcribed *in vitro*. Termination at methylated sites occurs one nucleotide before the corresponding dideoxy termination product. The right panel shows a control experiment from a gel that was exposed under identical conditions in which the sample was not treated with DMS before reverse transcription. The RNA template region and the template boundary element are highlighted. (**B**) Preincubation of a DNA oligonucleotide substrate with telomerase before DMS modification in the absence of deoxynucleotide triphosphates (dNTPs). Base formation is indicated and inferred from the protection of seven bases of the telomerase RNA template. Note the particularly high accessibility of the base in the active site at the +1 position relative to the DNA substrate. (**C**) Primer extension on addition of dNTPs. The bases that are protected after primer extension are indicated.

also shows a strong pause in the reverse transcriptase reaction of unmethylated RNA (supplementary Fig 1 online). Termination at this position may therefore reflect an *in vivo*-modified RNA base.

Speculation

Here, we assess the telomerase RNA structure in the catalytically active yeast Tlc1–Est2 core complex. The accessibility of Tlc1 to DMS supports the recently proposed secondary structure models (Dandjinou *et al*, 2004; Zappulla & Cech, 2004). The discrepancy between DMS accessibility and predicted secondary structure in the pseudoknot domain might be reconciled with structural changes that may occur during substrate translocation, which was not resolved here.

Our results demonstrate that the analysed telomerase core allows the formation of only 7 bp for primer binding and after primer extension. Thus, our work provides direct proof for previous proposals that the TERT may limit the number of base pairs during the reaction cycle (Hammond & Cech, 1998). Product DNA disengagement from template RNA bases 3′ of the active site during elongation should facilitate telomeric DNA substrate dissociation and/or translocation (Fig 3). Although yeast telomerase only inefficiently dissociates or translocates from elongated primers *in vitro*, primer dissociation or translocation clearly occurs *in vivo* where many telomeric repeats can be added in a single cell cycle (Teixeira *et al*, 2004).

RNA–DNA helix unwinding seems to be a unique feature of the TERT when compared with the related retroviral reverse transcriptases, which are known to form several hundred nucleotide-long RNA–DNA hybrid molecules during reverse transcription. Conversely, RNA polymerase II also limits the length of the RNA–DNA hybrid to 9–10 residues by interaction with a set of protein loops (Westover *et al*, 2004). Whether the TERT polypeptide functions in a similar way remains to be seen. A concomitant accessibility of bases to DMS and inaccessibility to base pairing (Fig 1) could also be explained by a model in which a sterically constrained RNA template is under tension after primer binding, allowing the formation of only three-quarters of a helical turn (Fig 3).

METHODS

Strain construction. Est2 was overexpressed from the Gal promoter as N-terminal His-tagged fusion protein. A plasmid coding for a PGAL1–His6–TEV cassette was constructed, by PCR-amplifying DNA fragments from pFA6a–TRP1–PGAL1 and pFA6a–HIS3MX6–PGAL1 (Longtine *et al*, 1998) with oligonucleotides His6–TEV–R2 (5′-GCGAATTCTCCTCCGGATTGGAAGTACAGGTTCTCACCAGAACCATGGTGATGGTGATGGTGCGATCCTCTCATTTTGAGATCCGGGTTTT-3′) and F4-short (5′-CGGAATTCGAGCTCGTTTAAAC-3′). The products were cloned into pBluescript and sequenced. The plasmids, named pKF13 (His3MX marker) and pKF14 (TRP1 marker), can be used to construct PCR-based P_{Gal}–His–TEV integration cassettes analogous to previously described plasmids (Longtine *et al*, 1998). The amino-acid sequence appended to the N terminus of the tagged protein is MRGSHHHHHHGSGENLYFQSGG. The primer sequence for amplification is 5′-(gene-specific sequence)-TCCTCCGGATTGGAAGTACAG-3′ (Longtine *et al*, 1998). Here, the *EST2* gene was tagged by amplifying pKF13 with DNA oligonucleotides F4-Est2

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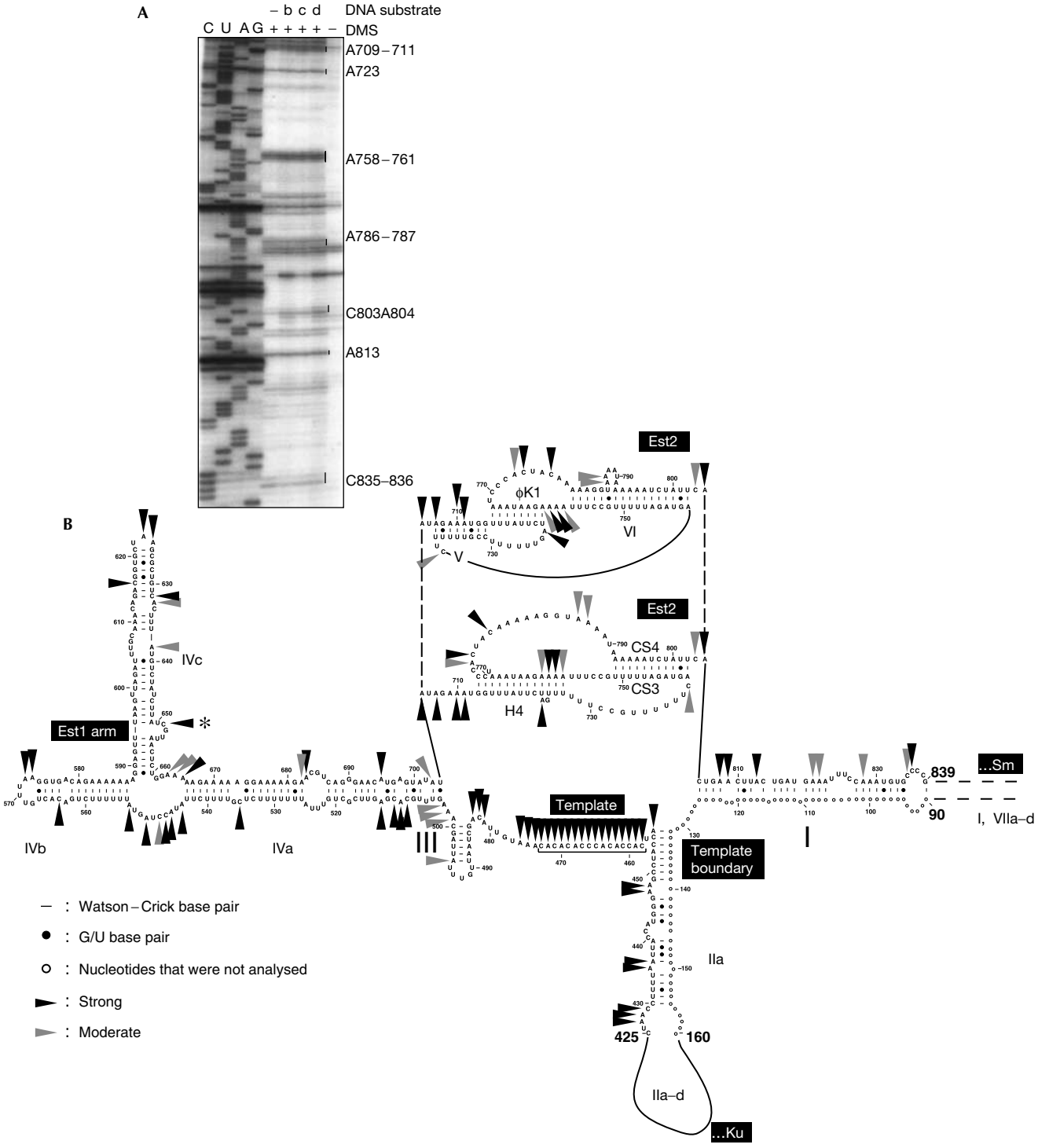

Fig 2 | DMS accessibility of the essential region of the telomerase RNA. (**A**) Est2-binding region. Some of the modified adenines and cytosines are highlighted on the right. DNA oligonucleotide substrates that were included in some of the reactions are b: 5′-GTGTGTGTGTGTGT-3′, c: 5′-GTGTGTGTGGG-3′ and d: 5′-TGTGTGTGGGTGTGGTG-3′. (**B**) Sites of DMS modification of the telomerase RNA superimposed on the *Saccharomyces cerevisiae* telomerase RNA secondary structure (Dandjinou *et al*, 2004; Zappulla & Cech, 2004). The upper pseudoknot structure is according to Dandjinou *et al* (2004) and the lower pseudoknot structure is according to Lin *et al* (2004). Only those bases of the RNA that were analysed in this study are shown. Strong modifications are indicated by black arrowheads and moderate modifications by grey arrowheads. Positions at which reverse transcription stopped also in the absence of DMS modification are ambiguous and are therefore not labelled. C651 (asterisk) in the bulge also shows a strong pause in the reverse transcriptase reaction of unmethylated RNA. Termination at this position may therefore reflect an *in vivo*-modified RNA base.

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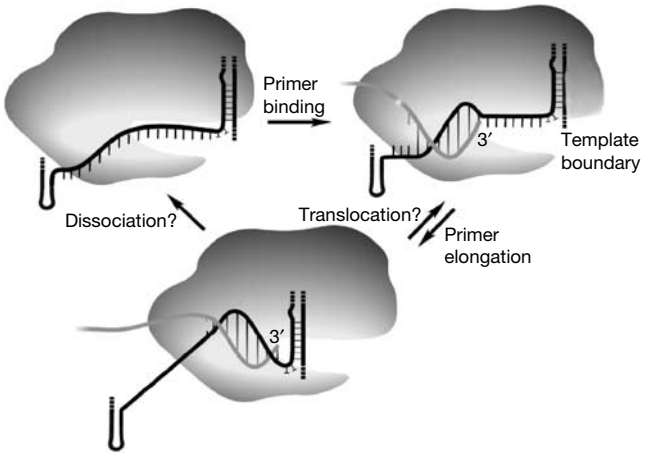

Fig 3 | Model for the telomerase reaction cycle. On substrate binding, a limited number of base pairs (seven in *Saccharomyces cerevisiae*) are formed between the 3′ end of the primer and the template region of telomerase RNA. During substrate elongation, base-pair formation at the DNA 3′ end coincides with base-pair disruption further 5′ on the DNA. Substrate elongation stops at or before the template boundary, which is imposed through a paired element in *S. cerevisiae* (Seto *et al*, 2003). Limitation of RNA–DNA duplex length should facilitate primer dissociation or translocation. Processive repeat addition is observed *in vitro* for telomerases from ciliates and vertebrates, but not from *S. cerevisiae*. The RNA template is modelled to be sterically constrained and under tension during primer binding and extension, allowing the formation of only three-quarters of a helical turn.

(5′-CACAAGTGAAATAGAAAAGTGAAAAATTAAAAAAAAAAAA AAAAAAAAAAAAAACTGATTTATACTCGAATTCGAGCTCGTTTA AAC-3′) and R6-Est2 (5′-TGTAGATCAATGTCAAGCTTGTCTT GAATGAACTCGAATAAGATTTTCATTCCTCCGGATTGGAAGTA CAG-3′) and by transforming FYBL1-4D (*MAT* **a** *ura3-Δ851 trp1Δ63 leu2Δ1 his3Δ200 lys2Δ202*).

The overexpression construct for the TLC1 RNA was obtained by subcloning the PGAL1–*TLC1* cassette from pRS314–PGAL1–*TLC1* (Forstemann & Lingner, 2001) into pRS306, yielding pRS306–PGAL1–*TLC1*. The plasmid was linearized with *Hpa*I and integrated at the *TLC1* locus in the strain above, leading to duplication of the *TLC1* gene with one *TLC1* gene under the control of the endogenous promoter and one under the control of the *GAL1* promoter. This strain is referred to as YKF24 (*MAT* **a** *ura3-Δ851 trp1Δ63 leu2Δ1 his3Δ200 lys2Δ202 HIS3MX6* P_{GAL1}*-HIS/TEV-EST2 TLC1::*P_{GAL1}*-TLC1 URA3*).

Yeast culture and telomerase preparation. YKF24 was precultured in galactose medium lacking uracil for 48 h at 25 °C to inoculate 2 l of rich galactose medium (2% peptone, 1% yeast extract, 3% galactose). Cells were grown at 25 °C to an OD_{600} of 1, collected by centrifugation, washed once with H_2O and resuspended in lysis buffer (150 mM Tris–HCl (pH 8.0), 150 mM NaAc (pH 8.0), 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.2% Triton X-100, 10% glycerol) supplemented with 2 × protease inhibitors without EDTA (Roche, Rotkreuz, Switzerland). Cells were lysed and telomerase was prepared by DEAE adsorption as described previously (Cohn & Blackburn, 1995; Prescott & Blackburn, 1997; Forstemann & Lingner, 2001). Most of the overexpressed Tlc1 RNA was associated with Est2 as determined by co-immunoprecipitation with a Myc-tagged version of Est2 (data not shown).

Telomerase RNA methylation. DMS modification was performed as described previously (Zaug & Cech, 1995; Antal *et al*, 2002). The reactions were carried out in 1 × telomerase reaction buffer (Forstemann & Lingner, 2001) in a volume of 20 µl containing DEAE-enriched telomerase fraction (20 µg of total protein). Substrate DNA oligonucleotides were included at a concentration of 25 µM. Substrate extension by the telomerase enzyme was initiated by addition of dNTPs (50 µM each) and incubation for 5 min at 23 °C. For RNA methylation, 1.5 µl of 10% DMS (diluted in 100% ethanol) was added to 20 µl reaction mixture, yielding a final concentration of 0.75% DMS. The samples were incubated for 5 min at 23 °C. The reactions were quenched by the addition of half volume of 2 M β-mercaptoethanol and incubated for 10 min at 23 °C. To digest the proteins in the methylated RNA sample, 200 µl of proteinase K solution (200 µg/ml proteinase K in reaction buffer) was added and the samples were incubated for 30 min at 23 °C. To acidify the solution, 25 µl of 3 M sodium acetate (pH 5.3) was added and the RNA was extracted with 150 µl of phenol/chloroform/isoamyl alcohol (25:25:1). The acid–phenol extraction partitioned DNA contaminations into the phenol phase. The aqueous phase containing RNA was transferred to a fresh tube containing 1 µl of glycogen solution (20 µg/ml) and precipitated with 600 µl of 100% ethanol. The RNA pellet was washed twice with 70% ethanol and resuspended in 10 µl of water. Dried gels were exposed for 1–3 days and signal strength was classified as strong or moderate visually, by both authors independently and discrepancies were re-evaluated by them together. All experiments were conducted at least twice, giving highly reproducible results.

Reverse transcriptase reactions and gel electrophoresis. The oligonucleotides used for primer extension by reverse transcriptase (tlcas600: 5′-GCAAATCTAACTTAAACTCC-3′ and tlcas 900: 5′-TAAGAAAGGACACCCTTGCC-3′) were labelled with [^{32}P-γ]ATP (equimolar amounts of ATP and oligonucleotide) and T4 DNA polynucleotide kinase (NEB) at their 5′ end. Labelled oligonucleotides were purified with the Nucleotide Removal Kit (Qiagen, Hombrechtikon, Switzerland). For reverse transcription, the entire 10 µl of methylated RNA was incubated with 1 pmol of radiolabelled oligonucleotide primer and 100 U of Superscript II reverse transcriptase (Invitrogen, Basel, Switzerland) according to the manufacturer's instructions. For parallel sequencing reactions, 1 pmol of the same radiolabelled oligonucleotide primer was used in standard dideoxynucleotide sequencing reactions (Amersham, Otelfingen, Switzerland) using pSD107 (Singer & Gottschling, 1994) as a template. After primer extension, all reaction products were precipitated with sodium acetate and ethanol according to standard protocols and resuspended in 5 µl of formamide loading buffer (90% formamide, 0.1 × TBE). The reaction products were separated on 6% acrylamide/urea gels according to standard procedures. The gels were dried and exposed to Kodak Biomax film for 2–14 days.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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