Specific features of telomerase RNA from *Hansenula polymorpha*

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ABSTRACT

Telomerase, a ribonucleoprotein, is responsible for the maintenance of eukaryotic genome integrity by replicating the ends of chromosomes. The core enzyme comprises the conserved protein TERT and an RNA subunit (TER) that, in contrast, displays large variations in size and structure. Here, we report the identification of the telomerase RNA from thermotolerant yeast *Hansenula polymorpha* (HpTER) and describe its structural features. We show further that the *H. polymorpha* telomerase reverse transcribes the template beyond the predicted boundary and adds a nontelomeric dT in vitro. Sequencing of the chromosomal ends revealed that this nucleotide is specifically present as a terminal nucleotide at the 3′ end of telomeres. Mutational analysis of HpTER confirmed that the incorporation of dT functions to limit telomere length in this species.

Keywords: telomerase RNA; telomere biogenesis

INTRODUCTION

Telomerase is a unique enzyme, which is capable of synthesizing telomeric DNA, templated by its own RNA subunit; the main function of this complex is to maintain telomere length (Blackburn 2000). The core components—telomerase reverse transcriptase (TERT) and telomerase RNA (TER)—are sufficient to maintain in vitro telomerase activity, although in vivo additional cellular factors are required for proper function of the enzyme (Collins 2006). TERT is known to be an evolutionary conserved reverse transcriptase (Lingner et al. 1997). TER varies greatly among species and its size ranges from 150 nt in ciliates to 450 nt in vertebrates and reaches 2000 nt in yeast (Theimer and Feigon 2006; Kachouri-Lafond et al. 2009). Mutational, structural, and phylogenetic studies revealed the presence of a few elements of higher order structure, which are required for enzymatic functions among all species and are similar among related organisms (Theimer and Feigon 2006). However, TER primary sequence is difficult to align even between closely related species.

The short length of the template is compensated by the ability of the enzyme to translocate newly synthesized DNA repeatedly to the beginning of the template, a process termed repeat addition processivity (Lue 2004). This feature varies between telomerases from different species, especially for enzymes studied in vitro. For example, ciliate or human telomerase can add hundreds of telomeric repeats to the substrate; *Tetrahymena thermophila* enzyme is processive, but starting from the second repeat, the efficiency of synthesis is largely reduced; yeast telomerases are usually nonprocessive, with rare exceptions such as the *Saccharomyces castellii* telomerase complex (Greider 1991; Cohn and Blackburn 1995; Lue 2004).

We focused our research on a telomerase from the thermotolerant yeast, *Hansenula polymorpha*, also known as *Pichia angusta*, which is able to grow under temperatures >50°C (Levine and Cooney 1973); thus, one can expect that its components—both proteins and nucleic acids—will be more stable. *H. polymorpha* belongs to a limited number of methylotrophic yeast and taxonomically is a member of the *Saccharomycetaceae* family. A recently published analysis of the mitochondrial genome of *H. polymorpha* (strain DL-1), its content, and gene order revealed a close phylogenetic relationship between *H. polymorpha* and *Brettanomyces custersianus* and suggested that these species should be assigned to a separate genus rather than included in the polyphyletic genus *Pichia*
of *Pichia* (Eldarov et al. 2011). Telomeres of *H. polymorpha* were reported to consist of the highly regular pattern 5′-GGGTGGCGG-3′ repeated 18–23 times (Sohn et al. 1999), which is shorter than the telomeres in other known species. The fact that *H. polymorpha* stands at the crossroads between different yeast species makes it particularly interesting to identify its telomerase RNA and investigate telomerase properties.

In this study, we report the identification of the telomerase RNA subunit *HpTER*. We present the secondary structure model of *HpTER* and compare its functional elements with other known telomerase RNAs. Characterization of telomerase activity in *H. polymorpha* revealed that after generating a telomeric repeat in vitro the enzyme efficiently synthesized a dT nucleotide, which was not present in telomeres at this position. Direct sequencing of telomeric ends showed that in vivo dT is incorporated only at the very end of telomeres. We confirmed the importance of this event in vivo by mutating telomerase RNA and analysis of telomere length in mutant strains. Mutants, which did not provide a basis for the synthesis of terminal mismatching sequence, possessed extended and heterogeneous telomeres. These results imply that *H. polymorpha* telomerase utilizes reverse transcription of nontelomeric nucleotide to control telomere elongation.

**RESULTS**

**Identification of the candidate for *H. polymorpha* telomerase RNA**

Genome sequence data from *H. polymorpha* strain DL-1 were obtained [the complete DNA sequence of *H. polymorpha* DL-1 is available from Genbank (AEOI01000001.1–AEOI01000013.1)]. A search for a putative ortholog of known yeast telomerase RNAs in the *H. polymorpha* genome did not yield any candidate, which is consistent with the fact that the RNA subunit is evolutionarily variable. We therefore used a biochemical approach to identify the gene of interest. The experiment was based on the strategy described previously for the identification of *S. pombe* telomerase RNA (Leonardi et al. 2008; Webb and Zakian 2008). We used recombinant *H. polymorpha* TERT (Smekalova et al. 2012) to obtain antibodies for the immunoprecipitation of the telomerase complex. The *H. polymorpha* telomerase was isolated by partial purification of S100 extract derived from wild-type *H. polymorpha* cells on a DEAE sepharose column as described previously (Cohn and Blackburn 1995). For the visualization of telomerase activity in DEAE fractions, a classical direct assay was performed with the use of the 13 nt telomere-like primer HD1 (5′-GGGTGCGCCGGGTG-3′) constructed using the sequence data for the *H. polymorpha* chromosomal ends (Sohn et al. 1999). We detected RNA-dependent DNA polymerization products straight above the radio-end-labeled primer from DEAE fractions 8–10 containing 400–500 mM NaOAc (Supplemental Fig. S1A). Telomerase isolated by DEAE fractionation of the S100 extract was then further purified by immunoprecipitation with *HpTERT* antibody. Partial retention of the telomerase activity on the beads proved the robustness of the approach (Supplemental Fig. S1B). Total RNA was recovered from the fractions enriched in telomerase activity and subjected to reverse transcription from terSearch primer (5′-GGGTGGCGGG-3′) generated with the known telomeric sequences and telomerase direct assays described above. The DNA product obtained in the first step was treated with terminal transferase to produce a G-tail on the 3′ end of the newly synthesized cDNA. PCR was performed from polyC and terSearch primer, followed by cloning and sequence analysis. This strategy revealed a candidate sequence matching the HPODL_contig07 of the *Pichia angusta* DL-1 position 711047-771215. Analysis of the locus showed that this region did not overlap with conserved ORFs. The *PDD4* gene, functionally related to the Vps (Vacuolar protein sorting) family in *S. cerevisiae*, is located upstream of the candidate locus. Notably, a Vps homolog is also present at the 5′ region of the *S. pombe* telomerase RNA (Leonardi et al. 2008). Downstream from the putative *HpTER* gene (*Hansenula polymorpha* telomerase RNA), there is a conserved ORF showing high homology with *Pichia pastoris* aromatic amino acid aminotransferase 1.

**Knockout of the *HpTER* gene leads to telomere shortening**

To determine whether disruption of the *HpTER* gene caused the predicted ever shorter telomere (EST) phenotype, we made a knockout of the *HpTER* gene. *H. polymorpha* TERT knockout was used as a control. The DL1-l strain used in this study is haploid under normal conditions and is a leucine auxotroph. A large part of the *HpTER* gene, including the putative template region, was replaced with an *HpLEU2* marker (Agaphonov et al. 1994). In the case of *HpTERT*, a selective marker was introduced in the middle of the coding region, disrupting the protein synthesis. After isolation of the deficient strains, the DNA products of the predicted length were detected by PCR analysis, which confirmed the robustness of the integration (Supplemental Fig. S2). The phenotype of two independent Δ*HpTER* transformants, the wild-type and the *HpTERT* deficient strain, was observed over time. Loss of cell viability could be detected from the first streak on the plate, which represented around 20 generations after the deletion of the gene (Fig. 1A). *H. polymorpha* chromosomes initially possess short telomeres (140–190 nt) (Sohn et al. 1999). Starting from the second restreak, null cells from liquid culture were unable to give rise to new colonies on the plate (Fig. 1A). Post senescent survivors appearing for both types of mutant cells from liquid culture after several restreaks (Fig. 1A) are most likely to be a result of homologous recombination (Ilpm and Greider 2003; Kachouri-Lafond et al. 2009).

The telomere length in these strains was analyzed by Southern blot of terminal restriction fragments with a
The 5′ in vivo HpTER transcript is present in several forms (Supplemental Fig. S3A). Any colonies on the plate under transformation conditions date. The 5′ (wt) strain (lane blot analysis of terminal restriction fragments for DNA from wild-type (see Materials and Methods for the detailed description). (A) Deletion of either HpTERT or HpTER gene led to loss of cell viability, followed by senescence and appearance of survivors (Supplemental Fig. S3). Deletion of either HpTERT strain (lanes 1, 2, 3, 4, 6, respectively), and ΔHpTER strain (lanes 7–11: stalks 1, 2, 3, 4, 6, respectively). ΔHpTER#1 transformant was used in this experiment.

To confirm that this phenotype was the result of HpTER deletion itself, we cloned the HpTER gene, including 150 nt upstream as the native promoter into the shuttle vector. We inserted this construct into the ΔHpTER knockout strain. Cell viability as well as telomerase activity was restored in this strain (Supplemental Fig. S3).

After the introduction of a plasmid, colonies on the plate were present only in the case of functional telomerase RNA on the plasmid; an empty vector without HpTER gene or with a nonfunctional mutant HpTER gene did not yield any colonies on the plate under transformation conditions (Supplemental Fig. S3A).

FIGURE 1. Analysis of the HpTER gene that encodes telomerase RNA in H. polymorpha. (A) Deletion of either HpTERT or HpTER gene led to loss of cell viability, followed by senescence and appearance of survivors (see Materials and Methods for the detailed description). (B) Southern blot analysis of terminal restriction fragments for DNA from wild-type (wt) strain (lane 1), ΔHpTERT strain (lanes 2–6; stalks 1, 2, 3, 4, 6, respectively), and ΔHpTER strain (lanes 7–11: stalks 1, 2, 3, 4, 6, respectively). ΔHpTER#1 transformant was used in this experiment.

Hpter transcript is present in several forms in vivo

The 5′ end of HpTER was defined from the strategy applied before for the identification of the telomerase RNA candidate. The 5′ end, in three of six sequences, began at the same position 168 nt upstream of the putative template region; one clone started 170 nt upstream and two others began 119 and 120 nt upstream of the putative template region (Supplemental Fig. S4). In order to map the 5′ end more precisely, 5′ RACE was performed on total RNA. This resulted in eight clones, seven of which placed the 5′ end at 168 nt and one at 169 nt upstream of the putative template region.

To define the 3′ end position of HpTER, RACE strategy was applied. The total RNA was isolated from the cells under mild conditions, and half the RNA was treated with polyA polymerase to add a polyA tail to the RNA 3′ end. cDNAs were obtained from both samples with a primer that consisted of oligo-dT and an adaptor. The cDNA was then subjected to PCR with the adaptor primer and the second primer specific to telomerase RNA. PCR fragments were subcloned and sequenced (Supplemental Fig. S4). The majority of naturally polyadenylated products had a 3′ end at position 792 (10 of 18); the longest product corresponded to 816 nt. One of the sequences lacked a possible intron (highlighted in Fig. 2). Six of the 25 sequences with naturally nonpolyadenylated RNA had a 3′ end at position 689. The most abundant product (10 of 25) had the 3′ end located at position 680 (Fig. 2).

A similar approach was applied to identify a 3′ end of HpTER in DEAE-isolated telomerase fraction. Naturally, polyA-containing products belonged to the same RNA region, the longest form was 824 nt in length (Supplemental Fig. S4). The nonpolyadenylated forms were the same or shorter than in the previous case (672–680 and 584–590).

Hpter secondary structure model

We predicted an HpTER secondary structure by combining several approaches. The Mfold web server (Zuker 2003) was used to determine overall HpTER architecture. The output was then adjusted according to existing data on particular functional elements of yeast telomerase RNAs with the help of the S2S program (Jossinet and Westhof 2005). The results are shown in Figure 2.

Mfold prediction yielded two alternative structures for the putative template boundary element (TBE) (Supplemental Fig. S5A,B). To distinguish between them, we performed mutation analysis that confirmed TBE structure “A.” Two distinct mutations, GCG/GCG161 and GCG/GCG130, were made to destroy stem element, whereas the combination of mutations restored the helix (Supplemental Fig. S5A). In accordance with the model, cells transformed with mutant GCG/GCG130 displayed an Est phenotype, whereas the combination of those mutations restored cell viability (Supplemental Fig. S5C). In Figure 2, the putative stem structure confirmed by this analysis is displayed.

Putative splicing is shown in the TER model. To prove that predicted splicing determinants are functional, we mutated 5′-ss (G690A_TGAGT) and branch point site (TACTA_A725C) in the HpTER gene. Corresponding shuttle vectors were then transferred into a strain deficient in HpTER. In case
of the branch point mutant, we could not obtain any transformant (Supplemental Fig. S6A). The 5' ss mutant was viable; however, the amount of HpTER transcript measured by real-time RT-PCR in the strain was ten times lower compared to the wild type (Supplemental Fig. S6B).

Characterization of the telomerase activity from *H. polymorpha*

To identify the DNA sequence synthesized by the *H. polymorpha* telomerase, we performed a reaction in which one of the three nucleotides (dA, dT, and dC) was substituted by its dideoxy analog (Fig. 3A). For this experiment, the G4 primer [5′-(GGGTGGCG)₄-3′] was used. Extension of G4 primer in the presence of ddT or ddC in the reaction resulted in a premature stop at the position expected from the template sequence (Fig. 3A, lanes 4, 5). Accordingly, the addition of ddA in the reaction mixture did not change the primer elongation pattern (Fig. 3A, lane 3). The *H. polymorpha* telomerase acted in a nonprocessive manner in vitro and did not produce more than one repeat.

Since *H. polymorpha* is a thermotolerant organism, we addressed the question whether its telomerase in vitro is active at higher temperatures. Optimum growth conditions for these species are known to be 37°C. Reactions were performed with the G4 primer from 4°C to 70°C. The fractions containing telomerase and reaction mixtures were preheated for 2 min at the desired temperature. In spite of the fact that *H. polymorpha* strains are able to survive at 50°C, telomerase activity in vitro could be detected only up to temperatures of 35°C (Fig. 3B). In contrast, lower temperatures do not seem to affect enzymatic activity much, and even at 4°C, the telomerase produced elongation of the primer up to four nucleotides.

*H. polymorpha* telomerase reverse transcribes beyond the predicted template boundary in vitro

Comparison of the HpTER to the telomeric sequence revealed a region between 171 and 187 nt (more than two repeats),
which could serve as a template for telomere synthesis (Fig. 2).

To determine the exact region used as template, we mutated
C176 to adenosine in the HpTER gene. The mutated gene
was introduced into the shuttle vector, transferred into a yeast
cell, and purified telomerase from the strain was obtained.
When ddT was present in the telomerase assay, the prelimi-
nary stop could be clearly seen at the position correspond-
ing to the C176A mutation (Fig. 4A, lane 6). This result con-
formed that nucleotides 178–171 of HpTER are reverse tran-
scribed. Analysis of the nucleotide pattern added by the
enzyme showed that the telomerase added nine nucleotides
to the G4 oligonucleotide, although the template provided
only eight nucleotides complementary to the annotated tel-
omeric sequence in H. polymorpha. According to the telomeric
sequence, the primer G4 [5′-(GGGTGCGG)₄-3′] should have
been followed by the eight nucleotides 5′-GGGTGCGG-3′.
That was partially verified in the previous experiment (Fig.
3A), which showed that the fourth and seventh nucleotides
added were thymidine and cytidine, respectively.

To identify the additional nucleotide, we performed a re-
action with the primer HD1 (5′-GGGTGCGGTTGTG-3′),
which should align to the middle of the template region
(Fig. 4B), and different sets of nucleotides were used to reveal
the sequence added by the telomerase. In the presence of only
radio-labeled dGTP, one nucleotide was incorporated into the
DNA (Fig. 4B, lane 1); dGTP plus dCTP gave a pattern of
three nucleotides; the incorporation of the fourth nucleo-
tide took place only if dTTP was present in the reaction mix-
ture (Fig. 4B, lane 3). From this experiment, we concluded
that the added sequence was 5′-GGGTGCGG-3′ and that the last
incorporated nucleotide was reverse transcribed from adenine
170, which was positioned beyond the predicted boundary.

Telomerase cannot use the sequence produced in vitro

Since the H. polymorpha telomerase DNA elongation product
synthesized in vitro cannot align perfectly to the begin-
ing of the template region, we wanted to test whether H. polymorpha
can elongate the oligonucleotide corresponding to the sequence produced in
vitro. We used a G4t oligonucleotide [5′-(GGGTGCGCGGTTGTG-3′)]
that contains the non-telomeric thymidine at the end of the sequence. We observed
a lack of signal in lane 1 (G4t substrate) compared to lane 2 (telomeric G4 sub-
strate) (Fig. 4C), indicating the inability of the telomerase to use the DNA pro-
duced by in vitro reverse transcription.

Occurrence of the non-telomeric nucleotide at telomeres in vivo

To elucidate whether the sequence 5′-
GGGTGCGG-3′ was present in internal telomere regions,
we analyzed 87 reads from H. polymorpha whole genome
shotgun sequences that displayed from 13 to 20 telomeric re-
peats (Supplemental File S1). Among the approximately 1400
analyzed repeats there was not a single case of thymidine po-
siioned after 5′-GGG-3′. After the sequence 5′-GGG-3′, we
detected some heterogeneity involving different numbers
of Gs (6.7% and 4.5% of the telomeric repeats contained five
and three Gs at this position instead of the four encoded by
the telomerase RNA template).

Since the additional nucleotide was not found in the anal-
ysis of contigs obtained from genome sequencing, the special
procedure for determining the terminal sequence of the ends
of the chromosomes described previously (Leonardi et al.
2008) was applied. The direct pyrosequencing of chromo-
somal ends amplification products showed the presence of
nontelomeric dT at the very end of telomeres (Supplemental
File S7). The analysis of the data according to the procedure
described previously (Sfeir et al. 2005) is presented in Figure
4D. Ninety percent of reads had extra nontelomeric dT as the
last nucleotide of telomere sequences. Taken together, these
data show that in vivo the extra nontelomeric dT residue is
present only at the telomeric 3′ end.

Reverse transcription of the A170 nucleotide is
significant for telomere biogenesis in vivo

To investigate the functional importance of the incorporation
of the nontelomeric nucleotide at the very end of telomere, a
number of mutations were introduced in the HpTER tem-
plate region. First, we mutated A170 of the HpTER into two
other nucleotides. Mutation A170C in the HpTER gene
should result in synthesis of a sequence corresponding fully to the telomeric one (Fig. 5A). A170G should produce the incorporation of a nontelomeric deoxycytidine in analogy to what is observed in the wild-type situation. An *H. polymorpha* strain lacking the endogenous *HpTER* gene was transformed with the vector containing the mutated *HpTER*. In the case of A170C, we detected a decrease in the efficiency of reverse transcription of C170 in comparison with A170 in a wild-type telomerase (Fig. 5B, C).

Nevertheless, this strain could be used as a system to trace the fate of telomeres when the only sequence provided by telomerase is the telomeric one.

We analyzed telomere length by Southern blot in strains with mutated telomerase RNA (A170C, A170G), a wild-type strain and also a strain in which the *HpTER* knockout was compensated by the introduction of a plasmid containing wild-type telomerase RNA (Δ*HpTER*). The shift of the radioactive signal to higher molecular weight indicated an elongation of telomeres in cells with the A170C mutation in HpTER compared to control strains. Further, the widening of the signal shows that telomeres became more heterogeneous (Fig. 5D, lanes 5, 6, cf. to lanes 1–4). The A170G mutation, which produced nontelomeric dC incorporation, did not influence significantly telomere length and homogeneity (Fig. 5D, lanes 7, 8, cf. to lanes 1–4). We then made a double mutant in which the initial A170C mutation was preserved but with nucleotide C171 mutated to A. In the double mutant A170C/C171A (noted AC170CA), telomeres became homogeneous and their lengths were similar to wt telomeres (Fig. 5E, lanes 3, 4, cf. to lanes 1, 2). In the C178A mutant the end of the copied sequence of the telomerase RNA remains unchanged, but this mutation allows for the efficient rebinding of the 5′-GCGT-3′ telomeric end (Fig. 5G).

Indeed, Southern blot analysis of telomere length in the C178A mutant showed that telomeres became elongated and heterogeneous in the corresponding strain (Fig. 5E, lanes 7, 8, cf. to lanes 5, 6 for wt). Since high heterogeneity of bands in the Southern blots made it difficult to

FIGURE 4. Reverse transcription of the nontelomeric nucleotide beyond the predicted template. (A) Telomerase activity assay for wt (lanes 1–3) and C176A mutant strains (lanes 4–6). Lanes 1, 4: telomerase assay in presence of dNTP mixture. Lanes 2, 5: after RNase treatment. Lanes 3, 6: with ddTTP added to the reaction mixture. The position of incorporated nucleotide is indicated at the left side of the gel. Position of 5′-phosphorylated primer is shown at the right side of the gel. The scheme of the HpTER template region, sequence of the HpTER mutated in C176A position and G oligonucleotide aligned to the beginning of the template are represented alongside. Preliminary stop for C176A in case of ddT usage indicated that the HpTER gene served as telomerase RNA in *H. polymorpha*. Note that the primer was extended by 9 nt, whereas only 8 nt could be added according to the template sequence encoding the telomeric pattern, the extra nucleotide is marked as "?" in the scheme. (B) Analysis of the nature of the extra nucleotide reverse transcribed by *H. polymorpha* telomerase beyond the template. Telomerase activity assayed for wt enzyme: lane 1: with HD1 oligonucleotide in presence of dGTP only; lane 2: with a mixture of dGTP and dCTP; lane 3: with a mixture of dGTP, dCTP, and dTTP. The position of the 5′-phosphorylated primer is shown at the left side of the gel. The scheme shows the alignment of HD1 primer along the HpTER template and telomerase elongation product (in bold letters). Addition of dT corresponding to the reverse transcription of A170 nucleotide in the telomerase RNA template region was revealed in lane 3. (C) Telomerase activity assay for wt telomerase with G4 primer (lane 1) and G4t primer (lane 2). (LC) loading control. The position of the primer alignment along HpTER template as well as the elongation product (in bold) is shown in the scheme. (D) Analysis of the sequences of terminal telomeric PCR products. In total, 1248 terminal telomere repeats were analyzed. The percentage for each terminal nucleotide is shown on the diagram.
estimate the actual length of telomeres in the C178A mutant, an alternative approach for telomere length analysis was applied. We measured the telomere length by PCR amplification of particular telomere as it was done for terminal telomere sequencing. Telomere amplification products were then separated in the agarose gel (Fig. 5F). It is clearly seen that, for wt and the mutants A170G, AC170CA, the telomeres remain short and stable and that, for the mutants A170C and C178A, the telomeres became extended and heterogeneous. These results confirmed the importance of the reverse transcription of the nucleotide at position 170.
the nontelomeric A170 nucleotide for telomerase action and
telomere biogenesis in *H. polymorpha* in vivo.

**DISCUSSION**

**Analysis of the HpTER structure**

Previously, the telomerase reverse transcriptase from *H. polymorpha* was successfully expressed and purified (Smekalova et al. 2012). In this study telomerase RNA from *H. polymorpha* was identified; a secondary structure model, incorporating a number of conserved features, was derived (Fig. 2). A 3′ RACE analysis allowed us to isolate different forms of HpTER (Supplemental Fig. S4). Most likely, long polyadenylated forms (between 792 and 824) represent primary transcripts. HpTER transcript polyadenylation is in agreement with the fact that the telomerase RNA moiety usually contains a portion of polyadenylated forms (Chapon et al. 1997). Short forms (680–689) were not polyadenylated and may result from HpTER processing.

Similar to the TLC1 RNA, the HpTER secondary structure model contains three helical arms (Fig. 2). A putative pseudoknot (Fig. 2), containing a triple helical element essential for telomerase RNA functioning (Theimer and Feigon 2006), covers nucleotides 368–457 and includes the nucleotides 396–399 (5′-AUGA-3′) of the CS3 hairpin conserved in all studied telomerase RNAs in fungi (Gunisova et al. 2009; Qi et al. 2013).

An important feature of *S. cerevisiae* TER is the Est1 binding hairpin located between the pseudoknot and the template region (Seto et al. 2002). In the HpTER secondary structure model, a putative Est1 hairpin is formed by nucleotides 252–332. Analysis of this element revealed the consensus sequences corresponding to the CS2 (5′-GAUGA-3′) and CS2a (5′-CUUCA-A-GG-3′) elements of TERs from *Candida* species (Fig. 2; Gunisova et al. 2009). A putative three-way junction element (TWJ) is formed by nucleotides 526–576. This putative structure contains the P6.1-like element of vertebrate TRs that is highly conserved between all the species (Brown et al. 2007; Qi et al. 2013) and found to be essential for telomerase activity in fungi (Qi et al. 2013). The L2 linker (single adenosine) and the first Watson-Crick AU pair in S3 hairpin are conserved in telomerase RNA of vertebrate and budding yeast, and they are present in the HpTER secondary structure model. In the case of HpTER, the putative linker L3 is made of nucleotides 565–GUCGA572 (Fig. 2), whereas it consists of 2 nt (5′-GU-3′) in most yeast and 4 nt (5′-GUCA-3′) in vertebrates (Gunisova et al. 2009).

It has been shown that the first step of splicing is essential for telomerase RNA biogenesis in fission yeast *S. pombe* (Box et al. 2008a). Splicing sites were detected in silico for telomerase RNA from *Candida* species, yet biochemically the significance of that splicing was not shown (Gunisova et al. 2009). The HpTER gene contains sequences that match the 5′ splice site (5′GUAGAGU3′) and the splicing branch point (720UACUAAC726) (Fig. 2, Supplemental Fig. S2); mutations in the putative 5′ splice site and branch point were significant for RNA maturation (Supplemental Fig. S6). RNA 3′ RACE analysis (Supplemental Fig. S2) also confirmed the predictions. One of the short abundant forms (689 nt) corresponded to the expected maturation product of the first splicing cleavage reaction. In *Hansenula*, the 3′ splice site consists of only 3 nt, which makes identification difficult (Blandin et al. 2000). Among the RACE clones, we were able to detect a full splicing product with an excised intron that allowed us to propose the position of the 3′-splice site (5′CAU740). These data suggest that the mechanism of HpTER processing includes participation of the spliceosomal machinery in the same way that it takes place for *S. pombe* telomerase RNA (Kannan et al. 2013).

The Ku70/Ku80 binding hairpin, found in other yeast (Stellwagen et al. 2003), is absent in the predicted model. This is an important, yet genetically not essential, element that mediates the interaction between telomerase and telomeres in yeast during cell cycle. Its absence in *H. polymorpha* species agrees with the previous suggestion that the element is not evolutionarily preserved even in yeast telomerase RNAs (Egan and Collins 2012).

A conserved element in TER sequence in many yeast species is the Sm binding site at its 3′ end (Dandjinou et al. 2004). Yeast consensus for Sm site is “AU(5-6)Pu” (Jones and Guthrie 1990). The HpTER gene lacks this exact pattern, although it contains several potential sequences that could provide putative Sm site: 683UUUCCUG690 and 675AUCUCCUG680. Interestingly, no canonical Sm site could be detected in case of *N. crassa* telomerase as well (Qi et al. 2013).

The putative template boundary element was identified in the HpTER secondary structure model (Fig. 2) and the necessity for cell viability of its secondary structure was confirmed by mutagenesis (Supplemental Fig. S5).

We showed that *H. polymorpha* telomerase reverse transcribes only 9-nt region (178–170) of the template (Fig. 4A, B). The adjacent segment of 9 nt, from 187 to 179, can potentially serve as a “primer-alignment” region. These elements are located in the single-stranded region adjacent to the putative TBE in the RNA secondary structure model (Fig. 2).

**Incorporation of nontelomeric dT to the very end of telomere mediates telomere length control system**

*Hansenula polymorpha* telomerase adds a nontelomeric nucleotide during telomere synthesis in vitro and in vivo (Fig. 4). Not all telomerases reproduce an exact telomerase RNA template sequence, and this results in telomere heterogeneity as detected in protozoa, some yeast species, slime molds, and plants (McCormick-Graham et al. 1997; Wellinger and Sen 1997; Förstemann and Lingner 2001). The divergence in telomeric repeats is most strikingly observed in *S. pombe* and *S. cerevisiae*. The causes for this degeneracy include multiple primer alignment registers in the RNA template, abortive
reverse transcription, the slippage of the substrate on the RNA template, and the low fidelity of the enzyme (Förstemann and Lingner 2001; Leonardi et al. 2008). Such heterogeneity can be also observed in internal parts of *Hansenula polymorpha* telomeres as revealed by the analysis of reads from *H. polymorpha* whole genome shotgun sequences. However, the incorporation of a nontelomeric nucleotide at the very end of telomeres by *H. polymorpha* telomerase both in vitro and in vivo (Fig. 4) is not due to mechanisms mentioned above but to the reverse transcription of A170 present in the telomerase template.

There are a number of examples when telomerase reverse transcribes beyond the template, but all these events are associated with mutations in telomerase components. Indeed, disrupting interactions in the TBE in different TERs can lead to transcription of the sequence following the template, rearrangement of the telomerase RNA structure, and a change in the processivity of telomerase (Seto et al. 2003; Box et al. 2008b; Booy et al. 2012). TERT is known to contribute to stabilization of the template boundary element through direct interactions in different species including *S. cerevisiae*, *T. thermophila* (Miller et al. 2000; Lai et al. 2002; Seto et al. 2003). Mutations in the C-terminal domain of hTERT were shown to mediate template definition; partial deletions of the hTERT C terminus resulted in reverse transcription proceeding beyond the template (Moriarty et al. 2005). However, the results obtained so far for wild-type telomerases have not provided evidence for reverse transcription of a nontelomeric sequence in vitro or in vivo. The nontelomeric dT could be incorporated during each elongation step and should then be removed for further productive telomere elongation as the inability of telomerase to elongate the nontelomeric 5'-GGGTGGCG-3'GGGTGGCT-5' sequence in vitro or in vivo. The nontelomeric dT could be incorporated during each elongation step and should then be removed for further productive telomere elongation as the inability of telomerase to elongate the nontelomeric 5'-GGGTGGCG-3'GGGTGGCGT-5' sequence in vitro of telomeres indicates.

Nuclease activity is known to be associated with human and yeast telomerase (Petrov et al. 1998; Huard and Autonier 2004). This nuclease activity can be responsible for eliminating the nontelomeric nucleotide after every round of telomeric repeat synthesis. Alternatively, an extra nucleotide could be added only at the very end of telomere when telomerase is switched to a nonprocessive mode. It should be mentioned that a similarly important functional activity was previously discovered for *Tetrahymena* telomerase. In that case, an extra nucleotide can be added, followed by a switch of telomerase to a nonprocessive mode and then removal through its 3'-5' nucleolytic activity (Collins and Greider 1993).

The fact that 90% of telomere ends contain the nontelomeric dT nucleotide indicates that this extra nucleotide serves as a marker for the end of a chromosome that has just been subjected to processing by telomerase and may be involved in telomere capping.

We showed that the reverse transcription of A170 resulting in the incorporation of a nontelomeric nucleotide at the very end of telomeres had an impact on telomere length in *H. polymorpha* yeast (Fig. 5). The strain with the A170C mutation in the telomerase RNA, that yields a product fully complementary to the telomeric sequence, possessed elongated and heterogeneous telomeres compared to the wild-type strain. Notably, the A170G mutation led to the incorporation of another nontelomeric nucleotide. In this mutant, telomeres remained homogeneous with length kept at its initial level. Also, the double mutation AC170CA provided telomeres close to the wild-type strain. These results indicate that telomere elongation in A170C resulted from changes in the DNA sequence produced by telomerase rather than from changes in the enzyme structure. It also implies that the incorporation of a nontelomeric nucleotide at the end of the synthesis round is part of the telomere length control system. Indeed, we were able to impair this system by another mutation (C178A). In this mutant, we introduced the possibility for a newly synthesized telomeric repeat with an extra dT at the very end to become fully complementary to the template region of TER. That immediately resulted in the appearance of highly heterogeneous and extended telomeres (Fig. 5). Taken together, our data allow us to propose that the incorporation of a nontelomeric nucleotide at the telomere end is a part of a system for telomere length maintenance in *H. polymorpha*.

The control of telomere length is complicated and varies with species. It involves cooperation between telomere maintenance, elongation, and shortening (Gilson and Géli 2007). A number of genes are found responsible for the mediation of the telomere length in various organisms. Regulation is usually associated with action of subtelomeric and telomeric protein factors that regulate telomerase activity in vivo (Smogorzewska and de Lange 2004; Schoeftner and Blasco 2009; Nandakumar and Cech 2013). The available data indicate that the binding of protein factors on telomere acts as a mechanical block, limiting accessibility of the substrate to the enzyme. Little is known about the participation of telomerase itself in the telomere length restriction. For *S. cerevisiae* it has been shown that the finger domain of the telomerase catalytic subunit is involved in negative control of the telomerase, because mutation in this region led to telomere lengthening (Eugster et al. 2006). Here, we provide evidence that the telomerase RNA contributes to the mechanism of telomere length restriction in *H. polymorpha* yeast. Considering the short length of *H. polymorpha* telomeres, it is possible that the organism has evolved in such a way that it developed a peculiar system of telomere length restriction. The possibility also exists that participation of the telomerase core complex in telomere length limitation is a common feature in other species.

**MATERIALS AND METHODS**

**Obtaining antibodies against HpTERT**

We generated polyclonal antibodies against HpTERT by immunization of the rabbit with the usage of Freund’s Complete Adjuvant according to manufacturers’ recommendations. Purified HpTERT
Telomerase isolation and assay

Purification of telomerase was performed as described (Cohn and Blackburn 1995). Briefly, cells were harvested in early log phase, disintegrated in liquid nitrogen, and resuspended in TMG50 buffer (10 mM Tris/HCl [pH = 8], 1.2 mM MgCl2, 10% glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM DTT, 50 mM NaOAc, RNasin [10 units/mL], supplied with the protease inhibitor cocktail complete mini [Roche Applied Science]). Derived S100 supernatant was subjected to ion exchange chromatography on DEAE sepharose (Biogel; Bio-Rad). We applied 30 mg total protein with concentration 0.25 mg/mL per 2 mL DEAE column equilibrated with TMG50 buffer, the gel was washed with 45 mL TMG50, and then NaOAc salt gradient (0.05—1 M 30 mL) was performed. In vitro telomerase assay was held under the following conditions: 50% DEAE fraction, 50 mM Tris/HCl (pH = 8), 1 mM spermidine, 1 mM DTT, 50 µM dNTP, 1 µM oligonucleotide, and 3.75 µM α-32PdGTP (800 Ci/mmol). The reaction products were analyzed on 10% denaturing PAGE. The markers were generated by radioactive labeling of the corresponding oligonucleotides using T4 PNK (Fermentas).

Identification of the telomerase RNA 5’ end

Total RNA was isolated as described in Hanna and Xiao (2006b). cDNA synthesis and PCR were performed using 5’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to manufacturer’s instructions. The deoxyribonucleotide qRF primer (5’-TGGACGTCCTCCATTTTGAC-3’) primed cDNA synthesis and was used as a forward primer in the PCR. The PCR products were cloned and sequenced.

Identification of the telomerase RNA 3’ end

Total RNA was isolated as described in Hanna and Xiao (2006b). A portion of RNA was polyadenylated using Poly(A) Tailing Kit (Ambion) according to manufacturer’s instructions. cDNA synthesis and PCR was performed using 3’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to manufacturer’s instructions. The qRF primer (5’-ATGCTGTCGGTGCTCCACT-3’) was used as a forward primer in the PCR. The PCR products were cloned and sequenced.

ΔHpTERT knockout strain phenotype assay and transformation

ΔHpTERT cells and ΔHpTERT#1 and #2 transformants (Fig. 1A) were grown overnight (first streak), diluted to equal density (OD600 = 0.02), and grown overnight again. Every day 4, serial fivefold dilutions of diluted cells (with OD600 = 0.02) were spotted on the YPD plate. This experiment was continued for 7 d (7 streaks).

To compensate HpTER knockout or to introduce mutated telomerase RNA into the ΔHpTERT knockout strain, we used shuttle vector pKAM556 with a modified Tn903 kanamycin resistance gene (Agaphonov et al. 2010). The HpTER gene was inserted into the pKAM556 by Sma1 site resulting in pKAM556_HpTER vector. For selection of G418-resistant transformants, 0.7× YPD supplemented with 120 mg/L G418 was used.

Cells from the stock made from a portion of ΔHpTERT cells (ΔHpTERT#2 transformant in Fig. 1A) after first streak were grown overnight and used for transformation performed with a standard protocol, modified as described (Bogdanova et al. 1995). Thus, on

Yeast strains and media

The taxonomy of H. polymorpha is still under discussion (Suh and Zhou 2010). In this work, we used two different strains of the H. polymorpha species. The HpTERT gene was cloned from ATCC 34438 (CBS4732) type strain of P. angusta (a.k.a. Ogataea polymorpha). Telomerase isolation and the telomerase RNA gene were derived from DL-1 (ATCC 26012) strain of H. polymorpha. Strains were kindly provided by M.O. Agaphonov (A.N. Bach Institute of Biochemistry RAS). Cells were grown on standard YPD media (1% yeast extract, 2% peptone, 2% glucose) under 37°C. To make knockout of HpTERT and HpTER, the LEU2 gene of H. polymorpha was used (Agaphonov et al. 1994), derived from pCHLX plasmid, kindly provided by M.O. Agaphonov. The HpTER gene was interrupted by the site Ste1 at the 1426 nt position, and the LEU2 gene was introduced inside the HpTERT gene, whereas for HpTER knockout, nucleotides from 55 to 563 were replaced with the LEU gene. Transformation of yeast with PCR product was performed with a standard protocol, modified as described (Bogdanova et al. 1995). Transformants, obtained with constructs containing LEU2, were selected on complete minimal medium without leucine (Hanna and Xiao 2006a); the integration was confirmed by PCR from genomic DNA (Supplemental Fig. S2).
a day of transformation, cells were after the second streak and could no longer yield any colonies on a plate (Fig. 1A), unless transformed with a plasmid with a functional telomerase RNA gene. Transformation with an empty vector without HpTER gene or a vector with nonfunctional mutant HpTER gene could not support formation of yeast colonies on the plate either (Supplemental Fig. S6A). Mutations were introduced in pKAM556_HpTER by QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene), and these products were sequenced to confirm mutations of interest and also the absence of secondary mutations in the HpTER gene.

qRT-PCR

HpU1 snRNA served as an internal control. cDNA was synthesized with HpTER-specific primer (qRr 5′-CCACCCACAGCGTGACTTC TC-3′) or with HpU1-specific primer (qHU1r 5′-ACTCCTCC GAAGGGAAGAG-3′) using RevertAid Reverse Transcriptase (Thermo Scientific) according to manufacturer’s protocol. Real-time PCR reactions were performed in a 25-μL mixture containing cDNA preparation, 1× Taq KCl buffer, 2.5 mM MgCl2, 0.6 μM primers, 0.2 mM dNTPs mix, 0.4× SYBR Green, and 1.5 units of Taq polymerase (Thermo Scientific). Primers for HpTER were qRf 5′-ATGCATTGCTGCTCCACTAAC-3′ and qRr 5′-CCACCCACAGCGTGACTTC-3′, Primers for HpU1 were qHU1f 5′-GGAGTCGACAGAGACGATG-3′ and qHU1r 5′-ACTCCTCCGAAGGGAAGAG-3′.

Southern hybridization

For Southern blot (Fig. 1B) ΔHpTER#1 transformant was used. Telomere length was analyzed by the standard Southern hybridization technique (Maniatis et al. 1982). Total chromosomal DNA was isolated and digested with EcoRI restriction endonuclease (Fermentas). After electrophoresis, the DNA was transferred onto a Hybond N+ (Amersham), nylon membrane. Hybridization was performed with the telomeric oligonucleotide G4 5′-(GGGTGG CG)3-3′ radiolabeled with α-32PdGTP by Terminal Deoxynucleotidyl Transferase (Fermentas).

Telomere PCR and sequencing

Genomic DNA was isolated as described in (Hanna and Xiao 2006b). DNA oligonucleotides PBoli733 [5′-GGTACAGCTCA CTGTAGATNNNNNN-3′-O(CH2)2CH2OH] and PBoli749 (5′-ACTCCTCCGAAGGGAAGAG-3′-biotin) were annealed at 100 nM in 20 mM Tris-acetate, pH 7.5, 5 mM NaCl and 2 mM MgCl2 by heating to 95°C and slow cooling to room temperature (Leonardi et al. 2008). The partial duplex (0.5 pmol) was then ligated to genomic DNA (1 μg) with T4 DNA ligase (Rapid DNA ligation kit, Thermo Scientific). PCR amplification of telomeres was performed with forward primer S12 (5′-AAGCGGGCACAGTGTTGG TTTCCAGGATGC-3′) and reverse primer PBoli745 (5′-GGGTA CGACTCAGTGTAG-3′) using GC-RICH PCR System (Roche). Cycling conditions were 3 min at 95°C; 0.5 min at 95°C, 0.5 min at 55°C, 0.5 min at 72°C (30 cycles); 10 min at 72°C. For telomere length, measurement by PCR PhiTurbo DNA polymerase (Stratagene) was used instead of DNA polymerase from the GC-RICH PCR System and 35 (instead of 30) cycles of PCR amplification.

The PCR fragments of telomeres from strain DL-1 of H. polymorpha were sequenced on a Roche GS FLX genome sequencer using the Titanium protocol. Sequencing reads that passed the quality filters, containing specific primer sequences, were extracted for further analysis.

DATA DEPOSITION

The DNA sequences of HpTERT and HpTER genes were submitted to GenBank under the following accession numbers: HpTERT JQ957926 and HpTER JQ957927.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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