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Humanized telomeres and an attempt to express a functional human telomerase in yeast

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ABSTRACT

The maintenance of telomeric repeat DNA depends on an evolutionarily conserved reverse transcriptase called telomerase. *In vitro*, only the catalytic subunit and a telomerase-associated RNA are required for the synthesis of species-specific repeat DNA. In an attempt to establish a heterologous system for the study of the human telomerase enzyme, we expressed the two core components and predicted regulatory subunits in the yeast *Saccharomyces cerevisiae*. We show that adequate substrates for human telomerase can be generated; the expressed enzyme was localized in the nucleus and it had the capacity to synthesize human-specific repeats *in vitro*. However, there was no evidence for human telomerase activity at yeast telomeres *in vivo*. Therefore functional replacement of the yeast telomerase by the human enzyme may require additional human-specific components. We also replaced the template region of the yeast telomerase RNA with one that dictates the synthesis of vertebrate repeats and performed a detailed molecular analysis of the composition of the telomeres upon outgrowth of such strains. The results suggest that vertebrate repeats on yeast telomeres are subject to a very high degree of repeat turnover and show that an innermost tract of 50 bp of yeast repeats are resistant to replacement.

INTRODUCTION

The ends of eukaryotic chromosomes, the telomeres, are essential to ensure genome stability and to facilitate the complete replication of chromosomal DNA (for recent reviews, see 1–3). Telomeric DNA in most organisms is composed of short direct repeats and a minimal amount of such repeats is essential to fulfil the telomeric functions (4). Telomeric repeat DNA consists of a species-specific length of double-stranded DNA and ends with a 3′-end overhang. In addition, owing to the sequence of the repeats, the strand composing the chromosomal 3′-end is usually rich in guanosines [referred to as the G-rich strand (5)]. As a consequence of the end-replication problem, the maintenance of a functional tract of telomeric DNA in most systems depends on a specialized reverse transcriptase, called telomerase (1,6). In human cells, telomerase activity is readily detectable in most transformed cell lines, cancer tissue cells and germ line cells, whereas there is little activity in normal somatic cells (for a review, see 7). This correlation between telomerase activation and tumorigenic transformation of cells has recently been corroborated with cell culture experiments (8). Thus the telomerase enzyme represents a promising and intensely studied target for anti-cancer therapies (9). In this respect, defined and tractable systems would be of great help to investigate the properties of telomerase.

The telomerase holoenzyme is a ribonucleoprotein (RNP) consisting of a number of proteins and an RNA molecule. The essential core elements, the catalytic protein subunit and the associated RNA, have been identified and cloned from a variety of organisms, including humans (hTERT, hTR) and the yeast *Saccharomyces cerevisiae* (Est2p, TLC1) (for reviews, see 10,11). *In vitro* studies of reconstituted human enzyme have shown that these two elements alone suffice to provide activity, but the experiments also indicated that additional factors may be needed for *in vivo* maturation, assembly, recruitment and/or activation of the RNP (12–15). Furthermore, genetic studies in yeast have identified several factors that are essential for *in vivo* activity of telomerase at the telomeres [Est1p, Est3p, Cdc13/Est4p and at least one ATM/ATR related kinase (16,17)], suggesting that the *in vivo* regulation of telomerase is much more complex than the current *in vitro* assays can recapitulate.

Insights regarding the *in vivo* regulation of human telomerase could be obtained by studying human telomerase in a heterologous system, such as yeast. Telomere structure and a number of the known telomerase-associated factors appear conserved between human and yeast cells. For

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example, the species-specific double-stranded telomeric repeats are bound by related proteins (scRap1p, hRAP1/TRF2, TRF1) and these proteins appear to regulate telomere length maintenance in both systems (18,19). In addition, there are clear homologies between the human and yeast catalytic proteins hTERT and Est2p, respectively (10). More recently, human proteins sharing similarities to the yeast telomerase-associated protein Est1p have also been identified, and the human hPOT1 protein may be a functional analogue of the yeast Cdc13/Est4p (for a review, see 20). In addition to these structural similarities, yeast telomerase will elongate telomeric substrates containing human repeats (21,22). Furthermore, substitutions in the yeast telomerase RNA template region to direct the synthesis of vertebrate-specific repeats results in telomeres containing vertebrate repeats (23). Such so-called humanized telomeres in yeast apparently are stable and the mitotic stability of the chromosome containing the human telomeric repeats is not affected (24,25). Finally, the human telomerase RNA can be stably expressed in yeast (26) and a telomerase activity synthesizing human repeats can be documented by immunoprecipitation from extracts of yeast cells coexpressing hTR and hTERT (27,28). However, despite the functional similarities of the telomere structures, telomerase and associated proteins between human and yeast, it remained unknown whether human telomerase could functionally complement the yeast telomerase in mediating telomere function and cell survival.

Here we report our efforts to reconstitute in yeast a functional human telomerase that is active on yeast telomeres. The results demonstrate that reprogramming the yeast telomerase RNA to template human repeats establishes telomeric end-structures comprising a relatively long 3′-overhang of the humanized G-rich strand. Therefore a suitable substrate for the human telomerase can be generated on yeast telomeres. Furthermore, we show that the expressed human telomerase subunits do form an active complex and localize to the nucleus. However, despite the presence of all these required prerequisites and the expression of two of the human hEST1 homologues in our yeast system, we were unable to detect any polymerization activity of the human enzyme on yeast telomeres.

MATERIALS AND METHODS

Plasmids and yeast strains

The pTLC1TRP and pTLC1hTRP plasmids were generated in the pRS314 backbone (29). First, pTLC1TRP contains a 2.9 kb NdeI–EcoRI fragment spanning the *TLC1* gene and isolated from pAZ1 (30) in the unique EcoRI site. Secondly, a 1 kb StuI–NsiI fragment of the *TLC1* gene in pTLC1TRP was replaced by the corresponding fragment isolated from pTLC1h (23). The resulting plasmid, pTLC1hTRP, thus contained the yeast *TLC1* gene with the template region converted to template human repeats. pEST2-LYS2 contains a 4.4 kb BamHI fragment with the *EST2* gene inserted into the SmaI site of pRS317 (31). The p413-hTR-ADE2 plasmid was created by replacing the original *HIS3* marker gene with the *ADE2* marker gene in p413-hTR (28). Plasmid pEGKT-hTERT (*URA3* marker gene) was described previously (28).

p426/CDC13$_{DBD}$-hTERT (*URA3* marker gene) was generated using an XbaI CDC13$_{DBD}$ fragment fused to a 3.4 kb XbaI–HindIII hTERT fragment from pEGKT-hTERT (28). The resulting SpeI–HindIII CDC13$_{DBD}$-hTERT fusion fragment was then cloned into the yeast expression vector p426-GAL1 (32) digested with SpeI and HindIII. pRS422-hTR (*ADE2* marker gene) was produced by cloning a SacI–XhoI fragment from p413-hTR (28) into the pRS422 vector (33) digested with SacI and XhoI. p425-HA2-hEST1A (*LEU2* marker gene) was constructed by inserting a PmeI restriction fragment containing HA2-hEST1A derived from pcDNA3.1-HA2-hEST1A (34) into p425-GAL1 (32). p424-HA2-hEST1B (*TRP1* marker gene) was constructed in the same way in p424-GAL1 (32). Note that the expression of the GST-hTERT, CDC13$_{DBD}$-hTERT, hEST1A and hEST1B proteins, as well as the hTR RNA, are under the control of the galactose inducible GAL1-promoter. When appropriate, proper plasmid constructs were confirmed by sequencing (fusion proteins and promoter insertions).

RWY12 (*Mat***a**, *ura3-52*, *lys 2–801*, *ade2-101*, *trp1-Δ1*, *his3-Δ200*, *leu2-Δ1*, *tlc1Δ::LEU2,* VR-*ADE2*-T) containing pAZ1 (*TLC1*, *URA3*) (35) was the strain used for the long-term telomere sequence replacement study. After selection of cells that had lost pAZ1 on 5-FOA plates, pTLC1TRP or pTLC1hTRP plasmids were introduced. One wild-type (pTLC1TRP-containing clone) and three independent pTLC1hTRP-containing clones, respectively, were restreaked on selective media for long-term sequence analysis (~2500 generations).

BY4705 (*Mat***a**/*Matα*, *ade2Δ::hisG/ade2Δ::hisG*, *his3Δ200/his3Δ200*, *leu2Δ0/leu2Δ0*, *lys2Δ0/lys2Δ0, met15Δ0/met15Δ0*, *trp1Δ63/trp1Δ63*, *ura3Δ0/ura3Δ0*) (33) was the host strain for the complementation study. *TLC1* disruption by KanMx4 was performed by one-step PCR gene replacement (33). The deletion of the *EST2* gene was performed by gene displacement using an EcoRI–BamHI fragment containing the *EST2* gene in which an internal 1.4 kb HpaI fragment was replaced by *HIS3*. The first 316 bp and the last 919 bp were homologous to the *EST2* gene. All deletions were confirmed by Southern blotting (data not shown). The resulting BY4705 *tlc1Δ::KanMx4/TLC1*, *est2Δ::HIS3/EST2* strain was then sporulated and selected spores deleted for both genes were first transformed with yeast telomerase plasmids pTLC1TRP (or pTLC1hTRP) and pEST2-LYS2 for maintaining survival. Then human telomerase plasmids pRS422-hTR or p413-hTR-ADE2 and pEGKT-hTERT or p426/CDC13$_{DBD}$-hTERT were introduced. Complementation tests were performed by loss of different plasmids and observation of survival of cells by replating on selective media. Strain YPH499 has been described previously (29).

Yeast and bacterial transformations and yeast genetic analyses were performed using standard methods (36–38).

Isolation and detection of telomeric DNA

Genomic DNA isolation, digestion with XhoI and gel analyses under non-denaturing conditions were described previously (35). Detection of single-stranded human telomeric repeats were performed with ^{32}P-labelled $(C_3TA_2)_n$ oligonucleotides. DNA was then denatured and transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech) and hybridized to

[^{32}P](C_3TA_2)$_3$ or [^{32}P](C_1-$_3$A) (5′-CCCACCACACACA-CCCACACCC-3′) oligonucleotides to detect double-stranded human and yeast telomeric repeats, respectively. For single- and double-stranded controls for human telomeric DNA detection, pTLC1hTRP was linearized by PsiI and either denatured prior to loading on the gel or used directly. pTLC1hTRP contains a tract of 19 bp of human telomeric repeats as targets. Alternatively, p16R containing 1.6 kb of human telomeric repeats was used (39). Linearized double-stranded and single-stranded phagemid DNA derived from pGT55 were respectively used as single- and double-stranded controls for yeast telomeric DNA (35). In all gels, end-labelled 1 kb ladder DNA (Gibco-BRL) was used as a DNA size standard.

Telomere cloning and sequence analysis

Chromosome V-R telomeres, which are linked to *ADE2* in the RWY12 strain, were amplified using an established protocol (40) with slight modifications. Briefly, genomic DNA from humanized cells was isolated after indicated numbers of generations of growth and resuspended in 50 µl of 5 mM Tris–HCl pH 8.0. Tailing of 3′-ends was performed by denaturing 100 ng of genomic DNA for 5 min at 95°C, after which tailing buffer complemented with 2 mM dCTP and 1 U of terminal deoxynucleotidyl transferase (TdT) (Amersham Pharmacia Biotech) was added and the reaction incubated for 30 min. The TdT enzyme was heat inactivated at 65°C for 10 min and at 94°C for 5 min. Tailing reactions were then transferred to puReTaq™ Ready-To-Go™ PCR Beads (Amersham Biosciences) containing the following primers at 0.8 pmol/µl: dG_{18}-BamHI (5′-CGGGATCCG_{18}-3′) and DIA5-1 (5′-GTGAGCGGATAACAATTTCACACAGTCTAGATGT-CCGAATTGATCCCAGAGTAG-3′). 3′-end amplification was performed with an initial denaturation at 94°C for 2 min followed by 45 cycles with 20 s denaturation (94°C), 12 s annealing (62°C) and 20 s extension (72°C), and a final extension step of 5 min at 72°C. PCRs were then run on 2% agarose gels and the bands were excised, purified and cloned into the pGEM-T vector (Promega). Plasmids were sequenced using M13 forward primer and SequiTherm EXCEL™II DNA sequencing kit on a LiCor 4200 DNA-sequencer. Sequencing data were analysed with GraphPad Prism software.

hTERT immunolocalization

Yeast cells used for the complementation study were grown to OD_{660nm} = 0.5 and fixed with 5% formaldehyde for 10 min. The cells were resuspended in PPBMG (100 mM potassium phosphate buffered at pH 6.5, 0.5 mM $MgCl_2$) plus 5% formaldehyde, incubated for 30 min and washed three times in B buffer (1.2 M sorbitol, 100 mM potassium phosphate buffered at pH 7.5). Rabbit anti-hTERT polyclonal antibody (1:200) (a generous gift from Lea Harrington) and monoclonal mouse anti-Nop1p (1:10 000) (41) antibodies were diluted in PBS containing 1 mg/ml BSA, applied to the cells and incubated for 2 h at room temperature. Cells were then washed several times with PBS containing 1mg/ml BSA. As secondary antibodies, Texas red conjugated goat anti-rabbit antibodies and Oregon green conjugated goat anti-mouse antibodies (Molecular Probes, Eugene, OR) were diluted 1:1000 and applied for 2 h to the cells. After several washes with PBS containing 1mg/ml BSA, DAPI (1 µg/ml in PBS) was added for 2 min and the cells were washed again three times with PBS. Slides were mounted and cells were visualized with an Olympus IX70 epifluorescent microscope.

Human protein expression, western analyses and telomerase activity

Expression of hTERT and hTR, determination of human telomerase activity and western analyses for the hTERT proteins were performed as described previously (27). Yeast strains containing either p425-HA2-hEST1A or p424-HA2-hEST1B plasmids were grown on selective medium containing 2% glucose or galactose to OD_{660nm} 0.5–3.0. Protein extracts were prepared as described (42). Samples were separated by 8% SDS–PAGE, transferred to Hybond C nitrocellulose membrane (Amersham) and probed with an Anti-HA mouse monoclonal antibody (Boehringer Mannheim), followed by secondary detection with horseradish peroxidase conjugated sheep anti-mouse antibodies (Amersham).

RESULTS

Previous reports suggested that a functional complementation of the yeast telomerase by the human enzyme may be possible. First, changing the template region of the yeast telomerase RNA into one that directs synthesis of human repeats results in mitotically stable yeast telomeres carrying human telomeric repeats (23–25). Secondly, the core subunits of the human telomerase can be expressed in yeast and assemble to form an active enzyme (27,28). However, none of these studies examined functional complementation of the yeast telomerase by expression of the human core components.

Creating the substrate and a molecular analysis of humanized telomeres

In vitro, ciliate telomerase requires single-stranded DNA with a 3′-end as a substrate (43). A 3′-overhang at the very distal end of chromosomal DNA is a conserved feature in many species, including yeast and humans (3). For telomerase-mediated elongation, the sequence of the overhang appears to be less important as long as there are some nucleotides to anneal with the template region. For example, yeast telomerase can use human and various ciliate repeat DNAs as substrates (21,22,44,45) and, at least *in vitro*, human telomerase can also use various substrates (46). To ensure that a suitable substrate for the human enzyme can be generated in yeast, we used a yeast telomerase RNA that carries human repeats at the template site (pTLC1hTRP). Previous reports showed that when this RNA replaces the endogenous wild-type yeast telomerase RNA, human telomeric repeats are incorporated into yeast chromosomal telomeres (23). We repeated this experiment and analyzed the incorporation of human repeats into telomeric DNAs and, in particular, into the 3′-overhangs. Single-stranded overhangs on telomeres can be detected using a non-denaturing in-gel hybridization technique, followed by detection of all telomeric repeat DNA in the same gel, once the DNA is denatured (35). Yeast cells expressing *Tlc1h* as the sole telomerase RNA were grown on plates for up to 2500 generations; genomic DNA was isolated at various intervals, digested with the XhoI restriction enzyme and analyzed as described above. Owing to a conserved

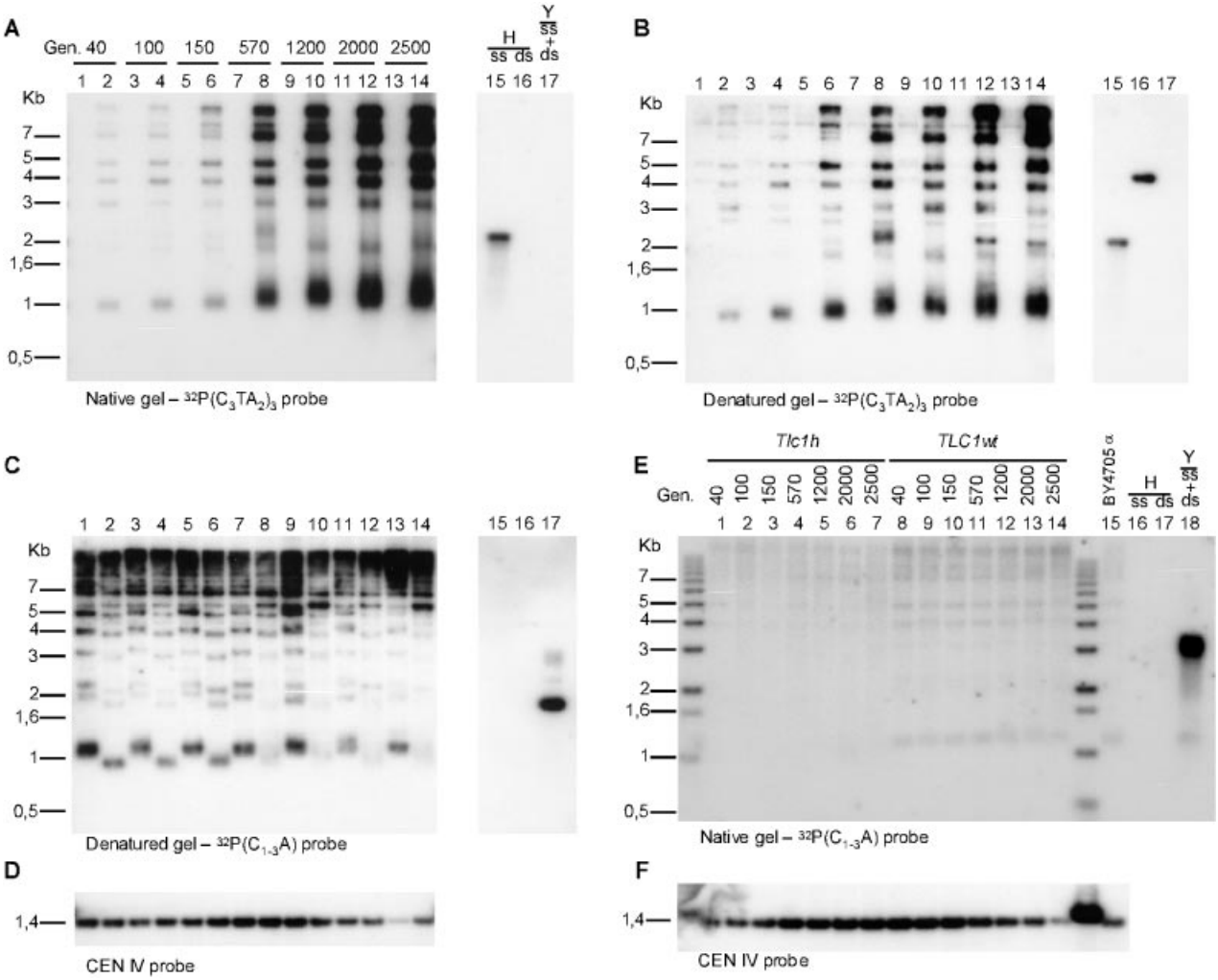

Figure 1. Single-stranded overhangs and sequence composition of telomeres in yeast strains expressing *Tlc1h*. Genomic DNA from yeast strains expressing wild-type *TLC1* and strains expressing the humanized RNA (*Tlc1h*) was extracted after the strains had been cultured for the indicated numbers of generations (40 to ~2500). The DNA was digested with *Xho*I and analyzed by non-denaturing in-gel hybridization. Lanes 1, 3, 5, 7, 9, 11 and 13: strains expressing *TLC1wt*. Lanes 2, 4, 6, 8, 10, 12 and 14: strains expressing *Tlc1h*. (**A**) The native gel was first hybridized to an end-labelled human telomeric probe [^{32}P](C$_3$TA$_2$)$_3$. (**B**) DNA in the gel shown in (A) was denatured and rehybridized to the same probe. (**C**) The probe in (B) was washed off and the gel rehybridized to an end-labelled yeast telomeric probe [^{32}P](C$_{1\text{-}3}$A). (**D**) The same gel as shown in (A)–(C) was rehybridized to a probe specific for the CEN4 region to show about equal loading of the lanes. (**E**) Non-denaturing in-gel analysis of the same DNA as shown in (A)–(D), but using the yeast-specific C-strand probe first. Lanes 1–7: strains expressing *Tlc1h*. Lanes 8–14: strains expressing *TLC1wt*. Lane 15: genomic DNA derived from a wild-type strain (BY4705) without any plasmids. (**F**) The DNA in the gel shown in (E) was denatured and rehybridized to a probe specific for CEN4 to control for DNA loading. Lanes 15–17 of the gel shown in (A)–(C) and lanes 16–18 of the gel shown in (E) contain control DNAs harbouring human telomeric repeats in single-stranded form (H, ss), human telomeric repeats in double-stranded from (H, ds) or a mixture of yeast telomeric repeats in single- and double-stranded form (Y, ss+ds). The DNA size standard was end-labelled 1 kb ladder DNA and sizes are indicated on the left of the gels.

subtelomeric Y′ element that is present on many yeast telomeres, XhoI liberates a diagnostic terminal restriction fragment (TRF) of about 1.2 kb, of which about 300 bp correspond to the terminal repeat DNA. Various other TRFs of larger sizes are also detected in this analysis and these correspond to non-Y′ telomeres (35). Single-stranded overhangs carrying human repeats of the G-rich strand can indeed be detected if a C-rich strand oligomer is used as a probe (Fig. 1A). Conversely, single-stranded C-rich DNA was never detected (data not shown). The signal detected corresponded to terminal extensions, since it was abolished if the DNA was treated with *Escherichia coli* Exonuclease I prior to analysis (data not shown). Intriguingly, the signal intensities for the human repeats increased with increasing numbers of generations, which suggested that the overhangs have a tendency to lengthen over time. In order to examine whether yeast-specific repeats are part of the overhang, the same DNA was also subjected to non-denaturing gel analysis using a yeast-specific C-strand probe. While a weak signal for G-strand extensions can be detected on DNA derived from wild-type *TLC1*-harbouring cells, no such signal was detected on DNA derived from *Tlc1h*-harbouring cells (Fig. 1E).

To examine the approximate size of the G-strand overhangs in these strains, we used probes of differing lengths and increasing washing stringencies with the non-denaturing in-gel procedure (39). The longest probe used (30 nt) remained

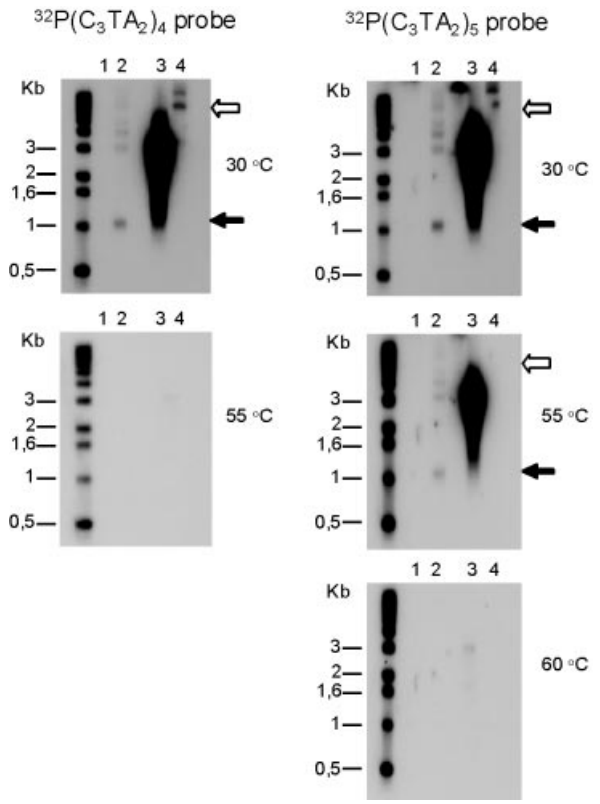

Figure 2. Vertebrate G-rich overhangs of at least 24 bases on telomeres of cells carrying *Tlc1h*. Non-denaturing in-gel hybridization was performed with human telomeric probes of various lengths. Shown here are the results obtained with $[^{32}P](C_3TA_2)_4$ (left) and $[^{32}P](C_3TA_2)_5$ (right) as probes. Gels were first washed at 30°C and, after appropriate exposures were obtained, they were rewashed at increasing temperatures as indicated on the sides of the panels. Lane 1: DNA derived from a strain carrying wt *TLC1* and grown for 100 generations. Lane 2: DNA derived from a strain carrying *Tlc1h* and grown for 100 generations. Lane 3: linearized and denatured p16R (positive control with 1.6 kb of vertebrate repeats). Lane 4: linearized and denatured pTLC1hTRP (positive control with 19 nt of vertebrate repeats). Left: molecular weight standard. Solid arrows indicate the chromosomal terminal restriction fragments (TRFs); open arrows indicate the pTLC1hTRP control.

hybridized to the DNA extracted from yeast to virtually the same extent as it remained hybridized to a positive control containing 1.6 kb of repeats (Fig. 2). However, the signals for a control single-stranded DNA containing a shorter tract (19 nt on pTLC1hTRP) were lost after washing the gels at 55°C (Fig. 2, right, middle panel, lane 4, open arrow). This suggests that the entire length of the probe could hybridize on G-rich overhangs, indicating that after culturing of about 100 generations, most humanized overhangs in yeast are >24 nt long.

After the non-denaturing analyses, the DNA in the gels shown in Figure 1A was denatured and consecutively rehybridized to probes detecting all human G- and C-strand repeats (Fig. 1B and data not shown). Consistent with what has been reported, TRF lengths of the *Tlc1h*-harbouring strains are shorter than wild type and remained quite stable for at least 2500 generations (Fig. 1B and C). However, signal intensities for human-specific repeats of both strands tended to increase over the course of the experiments (Fig. 1B and data not shown). Conversely, signal intensities obtained with a probe detecting all yeast-specific repeats clearly diminished in these samples (Fig. 1C). This apparent progressive replacement of yeast telomeric repeats with human repeats had been noted previously (24,25), but not examined in detail. We investigated this effect by cloning and sequencing the telomeric repeats adjacent to a marked telomere in the *Tlc1h*-harbouring strain at various times of outgrowth (Fig. 3). Overall, this sequencing effort confirmed the qualitative impressions from the hybridization experiments (Fig. 3B, composite bars). For telomeres containing both human and yeast repeats, the human repeats were distal to the yeast repeats in virtually all cases (data not shown) (23). In no clone did we find a complete replacement, however, even after 2500 generations. On all clones derived from cells grown for more than 1200 generations, a minimal tract of about 50 bp of yeast-specific repeats remained. Surprisingly, we found a significant number of telomeres that did not contain any human-specific telomeric repeats, even after 150 generations of growth in the presence of *Tlc1h*. As opposed to the telomeres harbouring a mixture of repeats, the telomeres with yeast repeats only decreased in size over time (Fig. 3B, white bars).

These results demonstrate that over the course of outgrowing yeast cells harbouring *Tlc1h* as the sole telomerase RNA, there is a progressive loss of the yeast-specific repeats on chromosomal telomeres. This loss is counterbalanced by a progressive increase of human repeats and overall sequence replacement is very slow, reaching a stable state only after 1200 generations. In addition, the single-stranded overhangs in such strains are composed of human G-strand repeats that are mostly >24 nt long, and these overhangs also appear to increase in size during outgrowth. Thus the data suggest that an adequate substrate for the human telomerase can be generated in yeast.

Expression and localization of the human enzyme

We next constructed a yeast strain that carried deletions of both the *EST2* and *TLC1* genes. Yeast cells devoid of telomerase activity are able to grow for about 50–70 generations, during which time telomeric repeat DNA is eroded (47). However, increasing telomere dysfunction leads to a growth arrest after such outgrowth. Therefore our starting strains also contained plasmids carrying *TLC1* and *EST2* (see Materials and Methods). For all assays, starting strains carrying pTLC1hTRP, the *TLC1* gene with humanized template, or pTLC1TRP, a wild-type copy of *TLC1*, in combination with the pEST2-LYS2 were used in parallel. Into these strains, we introduced yeast plasmids expressing hTERT as a fusion protein with GST (pEGKT-hTERT) or as a fusion protein with the Cdc13p DNA-binding domain (p426/$CDC13_{DBD}$-hTERT) in combination with an hTR-expressing plasmid. First, we assessed whether the human components were expressed and could reconstitute a detectable telomerase activity in yeast extracts (Fig. 4). Both the $CDC13_{DBD}$-hTERT and the GST-hTERT fusion proteins were expressed in yeast, since signals corresponding to their respective predicted molecular weights were detected by western analysis (Fig. 4B). In addition, combinations of hTR-expressing plasmids with plasmids expressing either hTERT-fusion protein resulted in detectable telomere repeat amplification protocol (TRAP) signals (Fig. 4A). Although the signals were not very strong, particularly when the $CDC13_{DBD}$-hTERT

A

number of generations	TG_{1-3} (nt) only telomeres	TG_{1-3} (nt) + T_2AG_3 (nt) telomeres		telomere length (nt)
40G	171 ± 13 (6/9)	129 ± 38	40 ± 5	169 ± 39 (3/9)
100G	96 ± 15 (5/14)	103 ± 14	43 ± 29	146 ± 26 (9/14)
150G	71 ± 14 (4/10)	90 ± 3	57 ± 29	147 ± 28 (6/10)
570G	0 (0/7)	87 ± 6	45 ± 31	131 ± 29 (7/7)
1200G	0 (0/7)	59 ± 5	124 ± 19	182 ± 22 (7/7)
2500G	0 (0/10)	48 ± 2	128 ± 36	175 ± 36 (10/10)

B

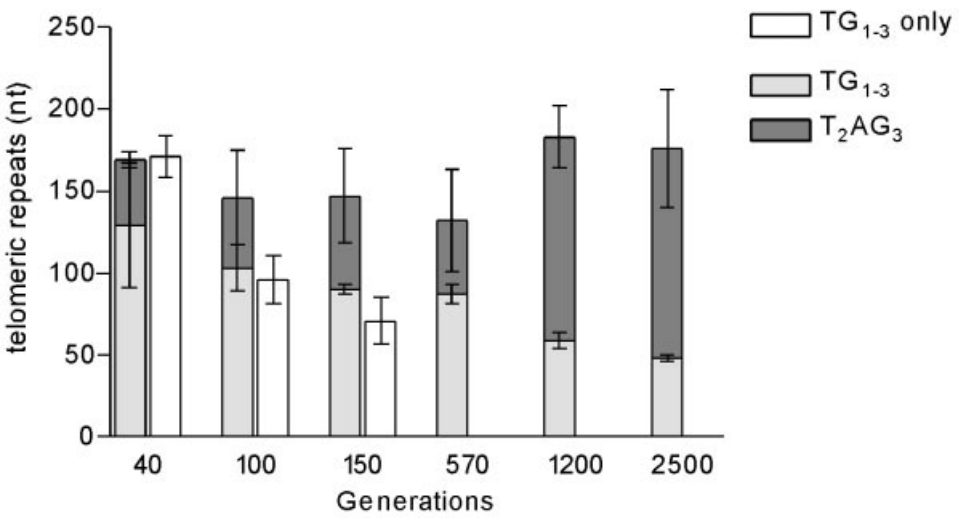

Figure 3. Sequence analysis of telomeres in *Tlc1h*-harbouring cells. (**A**) For each time-point indicated, three independent PCRs were performed, the amplified DNA was cloned and several independent clones were sequenced. The table indicates the average (± standard deviation) length of telomeric sequences obtained after a given number of generations of culture. The number of clones containing TG_{1-3} sequences only (middle column) or containing both TG_{1-3} and T_2AG_3 sequences (right column) per total clones sequenced is indicated in parentheses. Telomere length (far right) is calculated only for the mixed telomeres. (**B**) A bar graph representation of the data: white bars, telomeres with yeast sequences only; composite bars, telomeres with both human and yeast repeats. Note that the human repeats were always distal to the yeast repeats (data not shown).

construct was used, one must note that, in contrast with previous analyses (27,28), the assays were performed using crude yeast cell extracts. Moreover, according to the western analysis, $CDC13_{DBD}$-hTERT may be less efficiently expressed than GST-hTERT in yeast. We conclude that expression of either fusion protein in combination with hTR is able to yield an active human telomerase.

In vivo, telomerase substrates, or chromosome ends, are located in the nucleus; thus it was essential to assess whether the expressed human telomerase could be imported into the yeast nucleus. We localized the hTERT fusion proteins using indirect immunofluorescence and an antibody against hTERT (Fig. 5). Cells were also stained with DAPI as an indicator for the nucleus and an antibody against Nop1p, which marks the nucleolus. The hTERT antibody creates low levels of background signal in these assays (Fig. 5A). Clearly, both fusion proteins were found predominantly in the nucleus, yielding a spotted pattern throughout the nucleoplasm (Fig. 5B and C). Remarkably little of the protein remained detectable in the cytoplasm. Although we did not colocalize the hTR RNA, we assume that it is associated with the protein in the nucleus. Thus it is reasonable to suggest that the expressed human telomerase components can assemble into an active complex that localizes to yeast nuclei.

The human enzyme does not complement the yeast telomerase

Using our starting strains that contained the core components of human and yeast telomerase on four different plasmids, we selected for strains that had lost either of the components and assayed for *in vivo* complementation. Complementation of the yeast enzyme was scored as the ability of yeast cells to grow past the point at which telomerase-negative yeast cells encounter a growth crisis. Obviously, strains that retained both yeast components always displayed that phenotype, irrespective of whether they also contained other plasmids (Table 1). Such strains served as positive controls. Strains initially containing pTLC1TRP which had lost all four plasmids did display the expected growth arrest after about 70 generations and served as negative controls. Remarkably,

Table 1. Summary of complementation assays

Plasmids in WT strain	Plasmid loss	Survival (generations)	Plasmids in humanized strain	Plasmid loss	Survival (generations)
TLC1WT, EST2, hTR, hTERT	None	>150	TLC1h, EST2, hTR, hTERT	None	>150
None	hTR, hTERT, TLC1WT, EST2 (all four)	Death 70–90	None	hTR, hTERT, TLC1h, EST2 (all four)	Death <20
TLC1WT, EST2	hTR, hTERT	>150	TLC1h, EST2	hTR, hTERT	>150
TLC1WT	hTR, hTERT, EST2	Death 70–90	TLC1h	hTR, hTERT, EST2	Death <20
EST2	hTR, hTERT, TLC1WT	Death 70–90	EST2	hTR, hTERT, TLC1h	Death <20
TLC1WT, hTERT	hTR, EST2	Death 70–90	TLC1h, hTERT	hTR, EST2	Death <20
EST2, hTR	hTERT, TLC1WT	Death 70–90	EST2, hTR	hTERT, TLC1h	Death <20
hTR, hTERT	TLC1WT, EST2	Death 70–90	hTR, hTERT	TLC1h, EST2	Death <20
TLC1WT, EST2, hTR, $CDC13_{DBD}$-hTERT	None	>150	TLC1h, EST2, hTR, $CDC13_{DBD}$-hTERT	None	>150
hTR, $CDC13_{DBD}$-hTERT	TLC1WT, EST2	Death 70–90	hTR, $CDC13_{DBD}$-hTERT	TLC1h, EST2	Death <20

BY4705 cells carrying deletions of *TLC1* and *EST2* were transformed with yeast telomerase plasmids [pTLC1(h)TRP/pEST2-LYS2] in order to maintain survival. After sporulation, cells were transformed with plasmids carrying human telomerase subunit genes (pRS422-hTR or p413-hTR-ADE2/pEGKT-hTERT or p426/$CDC13_{DBD}$-hTERT). Complementation of yeast telomerase subunits by the corresponding human subunits can be assessed after loss of different plasmids followed by assessing survival of cells after restreaking on selective media. Columns 1–3 summarize the results obtained with strains harbouring pTLC1TRP (wt *TLC1*); columns 4–6 summarize the results obtained with the starting strain harbouring pTLC1hTRP (*Tlc1h*). Expression of human telomerase subunit genes was induced by growth on galactose-containing media.

strains that initially contained the pTLC1hTRP plasmid and thus contained the humanized telomeres before plasmid loss never yielded colonies without plasmids containing the genes for the yeast telomerase core components. We infer that growth arrest was occurring at less than 20 generations, and it is possible that such cells die extremely rapidly (Table 1, columns 1–3). This accelerated senescence could be attributed to the initially shorter length of the telomeres in these strains (Fig. 1) and possibly an accelerated loss of human telomeric repeats. As can be concluded from the results summarized in Table 1, following the loss of the plasmids for the expression of the yeast telomerase components, no combination of the remaining plasmids allowed the yeast cells to grow past the normal growth arrest point. Previous reports indicate that the action of yeast telomerase at the telomeres is independent of additional cofactors such as Est1p, when Est2p is fused to the Cdc13p DNA-binding domain (48). As we initially did not co-express human versions of such cofactors, we speculated that fusing hTERT with $CDC13_{DBD}$ might bypass a requirement for them. However, targeting hTERT to the telomeres by expression of a $CDC13_{DBD}$-hTERT fusion protein did not rescue a yeast telomerase deficiency. Of note, the $CDC13_{DBD}$-hTERT fusion protein used here does complement the temperature-sensitive growth defect of a strain harbouring a *cdc13–1* mutation, demonstrating that the Cdc13p included in $CDC13_{DBD}$-hTERT is functional (data not shown). In addition, chromatin immunoprecipitation experiments have shown that Cdc13p is bound to humanized telomeres to the same extent if not more than to wild-type telomeres (24). These data strongly suggest that the failure to provide human telomerase activity onto the humanized telomeres is not due to a general failure to target the hTERT protein to the telomeres. Finally, we also expressed hEST1A or hEST1A and hEST1B together with the core human telomerase components. Again, although the hEST proteins could be expressed from our yeast vectors, as assessed by western analysis (Fig. 4C), no complementation of the yeast telomerase deficiency was observed (Table 1).

We concluded that despite the expression of the two essential human telomerase core components in yeast, their assembly to form an active complex *in vitro* and the localization of hTERT (and presumably hTR) to the nucleus, human telomerase cannot functionally replace yeast telomerase.

DISCUSSION

The telomerase enzymes of particular species appear to have a relatively relaxed sequence requirement in terms of their substrates *in vitro* and *in vivo*. In addition, recent results showed that the yeast telomerase RNA can be reprogrammed to add vertebrate-specific repeats onto the ends of yeast telomeres (23–25). These features prompted us to ask whether a functional complementation of the yeast core components by the human counterparts was possible. Such an exogenous reconstitution system in yeast cells would be an invaluable tool for an in-depth analysis of telomerase function. However, none of the core component combinations tested in this study yielded evidence for a functional human telomerase activity at the endogenous yeast telomeres (Table 1). One could argue that the human telomerase enzyme can be active at yeast telomeres, but that the actual amounts of active RNPs reached in our experiments are too low for maintaining stable repeat tracts. We have shown previously that the co-expressed RNA and hTERT components do assemble into an active RNP with high specificity and that this active RNP can be purified by immunoprecipitations (28). Nevertheless, we also sequenced a number of telomeres cloned from the parent strain harbouring both telomerases. If there was a very low level of human telomerase activity on yeast telomeres, we would have expected to find occasional and perhaps isolated human repeats embedded in the yeast repeats. However, no such repeats were found amongst more than 1.5 kb of yeast telomeric sequences, representing more than 10 independent telomeres (data not shown). Given that the core components of human telomerase expressed in yeast can assemble to yield an

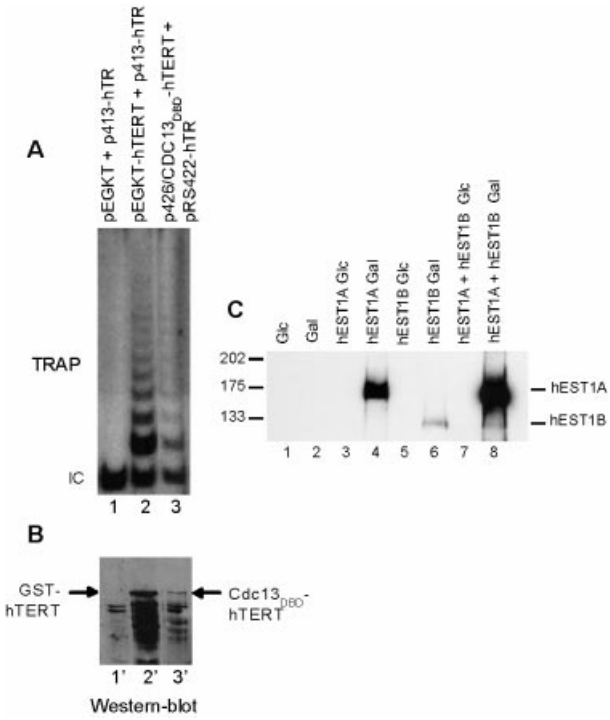

Figure 4. Detection of expressed human proteins and telomerase activity. (**A**) Telomerase activity was visualized by a TRAP assay using crude yeast extracts derived from cultures harbouring the indicated plasmids. Lane 1: YPH499 cells with pEGKT (empty vector) + p413-hTR. Lane 2: YPH499 cells with pEGKT-hTERT + p413-hTR. Lane 3: BY4705 *est2Δ/tlc1Δ* cells with p426/CDC13$_{DBD}$-hTERT + pRS422-hTR + pEST2-LYS2 + pTLC1TRP. IC, internal control signal for the PCR. (**B**) Western analysis using an affinity-purified antibody against hTERT was performed to detect GST-hTERT and CDC13$_{DBD}$-hTERT proteins (see arrows). Protein extracts analyzed were derived from the same cells as in (A). Note that the hTERT antibody does cross hybridize to some other proteins, but the bands at the predicted molecular weight for the fusion proteins (about 151 kDa for GST-hTERT and 155 kDa for CDC13$_{DBD}$-hTERT) are specific for the strains expressing them. (**C**) HA2-hEST1A and HA2-hEST1B expression as analyzed by western blot of total protein extracted from yeast strains grown in the presence of glucose or galactose. Anti-HA antibody was used as a probe and the expected molecular weights for HA2-hEST1A and HA2hEST1B are 166 and 122 kDa, respectively. Lanes 1 and 2: yeast strain BY4705 grown in glucose (Glc) or galactose (Gal) containing media (no plasmids). Lanes 3 and 4: yeast strain BY4705 with p425-HA2-hEST1A grown on indicated media. Lanes 5 and 6: yeast strain BY4705 with p424-HA2-hEST1B. Lanes 7 and 8: yeast strain BY4705 with p425-HA2-hEST1A and p424-HA2-hEST1B. The exposure time for lanes containing HA2-hEST1B and HA2-hEST1A + HA2-hEST1B (lanes 5–8) was longer than for HA2-hEST1A alone (lanes 3 and 4). About equal amounts of protein were loaded in each lane and molecular mass markers (in kDa) are indicated on the left.

enzyme with detectable activity *in vitro* (28) (Fig. 4), these results strongly suggest that additional cofactors are required for the assembly of an active enzyme at the site of synthesis.

The yeast Est1p and Est3p proteins are examples of such cofactors in yeast. While these proteins are not essential for initial cell growth and *in vitro* telomerase activity, they are essential for *in vivo* telomerase activity (49,50). In addition, one of the non-essential functions of the yeast Cdc13p protein is such a cofactor role (50). We addressed the requirement for cofactors by expressing an CDC13$_{DBD}$-hTERT fusion protein or by co-expressing two of the recently identified human *EST1* orthologues; however, these cofactors were not sufficient to reconstitute human telomerase function at yeast telomeres (data not shown). Therefore it is reasonable to suggest that additional components of the human enzyme might be necessary for *in vivo* activity of the enzyme at the heterologous telomere. These additional components could include known human telomere-associated proteins such as hPOT1 or the complexes assembled around the TRF1 and TRF2 proteins (18). Moreover, it is entirely possible that essential cofactors for *in vivo* activity of the human telomerase remain to be discovered. Lastly, regulation of human and yeast telomerase and telomere function may be sufficiently distinct to prevent functional action of human telomerase at yeast telomeres.

In vitro, the telomerase substrate sequence requirements are not stringent; however, we speculated that the human enzyme may require vertebrate-specific repeats *in vivo*. By reprogramming the yeast telomerase RNA, we found that a vertebrate-specific substrate can be generated in yeast and that the overhangs in such strains are mostly >24 nt long (Fig. 2). Upon long-term culturing, these overhangs apparently lengthen, thereby in principle providing an adequate substrate for human telomerase. While the essential yeast capping protein Cdc13p has a lowered affinity for single-stranded vertebrate repeats (51,52), it is able to bind them *in vivo*, and the relatively long repeat tracts reported here are consistent with the observed increase in Cdc13p binding at such telomeres (24).

The fine-structure analyses and the sequencing of telomeres in yeast strains expressing a humanized yeast telomerase RNA revealed a pattern of sequence replacement over many generations (Fig. 3). Yeast telomeric repeats are slowly and gradually replaced by human repeats in such strains, reaching a plateau after about 1200 generations. At this point, all telomeres sequenced still retained about 50 bp of yeast-specific repeats (Fig. 3), and all of these sequences encompass at least two Rap1p-binding sites (data not shown). Although recent results have shown that yeast telomeres containing only vertebrate repeats can be mitotically stable (24,25), complete replacement of yeast repeats appears not to occur *in vivo*, at least within 2500 generations of growth (Fig. 3). These results are consistent with telomeric repeat turnover experiments in another yeast, *Kluyveromyces lactis*, where a similar resistance to turnover for the innermost repeats has been observed (53).

Most intriguingly, our sequencing efforts also revealed that in such strains there are telomeres with about 70 bp of entirely yeast repeats even after 150 generations of growth in the presence of a humanized yeast telomerase (Fig. 3). In the absence of telomerase activity *in vivo*, yeast telomeres shorten by about 3–5 bp per end and generation, such that they reach a growth arrest due to deficient telomeres after about 70 generations (54). Therefore telomeres with only yeast repeats after 150 generations of growth in the presence of *Tlc1h* are difficult to explain solely with the premise that the humanized telomerase has never acted on them. It is possible that in the particular situation created here, telomeric sequence replenishment by recombination is more effective, which would result in a lower loss rate. Alternatively, the entirely yeast repeat telomeres found after 150 generations could be the result of consecutive sequence addition and sequence loss events. The added sequences would have been vertebrate

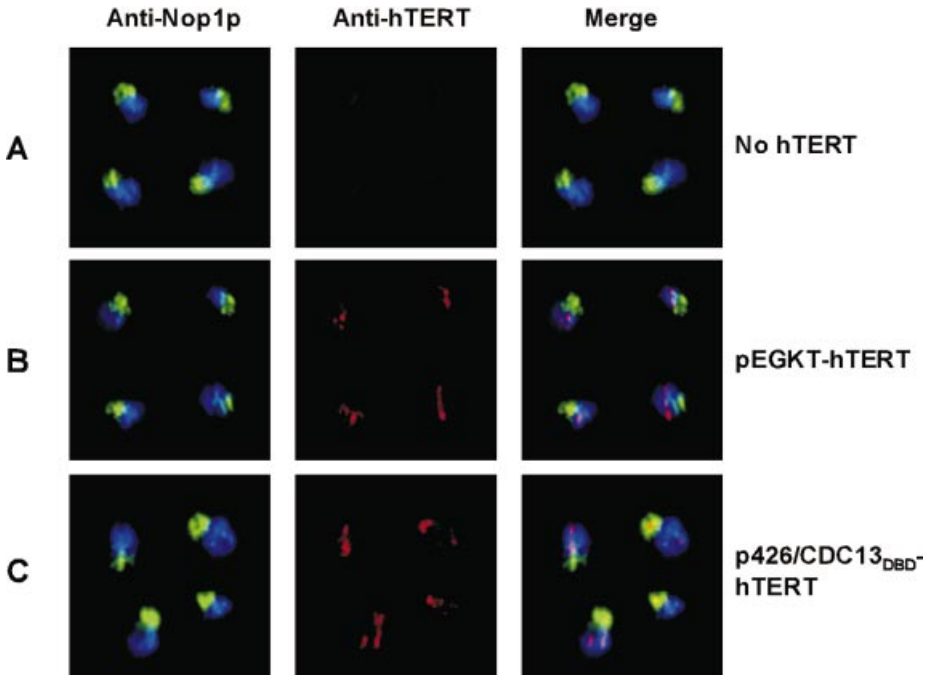

Figure 5. hTERT is localized to the yeast nucleus. In all panels, the nucleolar protein Nop1p was detected with a mouse monoclonal antibody against Nop1p and Oregon green-labelled goat anti-mouse antibody; hTERT fusion proteins were detected with a rabbit anti-hTERT polyclonal antibody and Texas red conjugated anti-rabbit antibody (red), and the nucleoplasm is represented in blue by DAPI staining.(**A**) control strain with no hTERT plasmid (BY4705 *est2*Δ/*tlc1*Δ cells with pEST2-LYS2, pTLC1hTRP and pRS422-hTR plasmids); (**B**) same as (A) plus pEGKT-hTERT plasmid; (**C**) same as (A) plus p426/CDC13$_{DBD}$-hTERT plasmid. Representative fields with four cells are shown and identical results were also observed using strains that harbored *TLC1wt* instead of *Tlc1h* (data not shown).

repeats, and while they were present the internal block of yeast repeats would have been protected from further gradual losses. Yet, in this scenario, the complete distal block of vertebrate repeats would have to be lost at a surprisingly high frequency to expose only the yeast repeats. One interpretation of the data would stipulate that telomere rapid deletion (TRD) occurs at an extremely high rate in these strains. TRD has been described as a one-step intrachromatid deletion event involving the distal single-stranded overhang and the proximal double-stranded portion of the telomeres (55). TRD is controlled by Rap1p-mediated telomere length control and the progressive losses of Rap1p binding sites from the telomeres in the *Tlc1h* carrying strain may allow an increased rate of TRD. In addition, the precision of TRD events is lost in *rap1^t* mutants, contributing to an increased heterogeneity of telomeric repeat tracts (55), a feature that we also observe during long-term culturing of cells (Figs 1 and 3). However, overall length heterogeneity is less pronounced than in *rap1^t* cells, arguing that Tbf1p, which will bind the vertebrate telomeric repeats (24), can impose a Rap1p-independent telomere length regulation (25). Finally, TRD occurs at quite high frequency during meiosis, even in wild-type cells (A. Lustig, personal communication). One could therefore invoke the possibility that catastrophic meiotic TRD may be the cause for the severe meiotic defects displayed by yeast strains carrying telomeres with vertebrate telomeric repeat tracts (24). Alternatively, the abrupt losses of the distal vertebrate telomeric repeats could be the result of direct degradation events. However, such degradation events appear to be rare in strains harbouring the yeast Ku-proteins and it has been reported that the yeast Ku-complex is still bound to telomeres harbouring vertebrate repeats (24). It could be argued that TRD- or degradation-mediated loss of vertebrate repeats would leave a few repeats at the yeast–vertebrate repeat boundary, which we should have detect by the sequencing effort. However, re-elongation of such shortened telomeres could be compared to telomere healing, and it has been shown that vertebrate repeats are very poor substrates for healing events by yeast telomerase (56). Therefore such ends may be subject to continued progressive sequence losses until all the vertebrate repeats are lost and the most effective substrate for re-elongation is exposed. Consistent with this idea, most efficient substrates for telomere healing include about 40–50 bp of yeast repeats comprising two Rap1p binding sites (56), and this corresponds well to what we found to be the obligatory sequences left on yeast telomeres after outgrowth in the presence of *Tlc1h* (Fig. 3). We therefore speculate that an innermost sequence of about 50 bp comprising two Rap1p binding sites is resistant to telomere turnover events and therefore may constitute the preferred minimal functional telomeric repeat tract for *S.cerevisisae*.

Taken together, the results reported here demonstrate that the core components of human telomerase can be expressed in yeast, are properly localized to the nucleus and assemble to form an active enzyme detectable in crude extracts. Moreover, relatively long single-stranded overhangs of vertebrate-specific repeats can be generated in yeast by reprogramming the yeast telomerase RNA, thereby providing the suitable sub-

strate for the human enzyme. However, the human enzyme is not active on yeast telomeres *in vivo*, even when targeted to the telomeres by fusing hTERT to Cdc13p or by co-expressing human orthologues of Est1p. Finally, telomeres carrying a mix of yeast and vertebrate-specific repeats may undergo frequent rapid deletions of the distal vertebrate repeats. These turnover events, however, never remove the entire yeast-specific tract and about 50 bp, encompassing two Rap1p binding sites, always remain at the proximal end. The system would therefore be ideally suited to study the mechanistic details for TRD and telomere sequence turnover, mechanisms that have been proposed to contribute significantly to telomere behaviour in mammalian cells (55).

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