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A human-*Tetrahymena* pseudoknot chimeric telomerase RNA reconstitutes a nonprocessive enzyme *in vitro* that is defective in telomere elongation

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ABSTRACT

The phylogenetically-derived secondary structures of telomerase RNAs (TR) from ciliates, yeasts and vertebrates are surprisingly conserved and contain a pseudoknot domain at a similar location downstream of the template. As the pseudoknot domains of *Tetrahymena* TR (tTR) and human TR (hTR) mediate certain similar functions, we hypothesized that they might be functionally interchangeable. We constructed a chimeric TR (htTR) by exchanging the hTR pseudoknot sequences for the tTR pseudoknot region. The chimeric RNA reconstituted human telomerase activity when coexpressed with hTERT *in vitro*, but exhibited defects in repeat addition processivity and levels of DNA synthesis compared to hTR. Activity was dependent on tTR sequences within the chimeric RNA. htTR interacted with hTERT *in vitro* and dimerized predominantly via a region of its hTR backbone, the J7b/8a loop. Introduction of htTR in telomerase-negative cells stably expressing hTERT did not reconstitute an active enzyme able to elongate telomeres. Thus, our results indicate that the chimeric RNA reconstituted a weakly active nonprocessive human telomerase enzyme *in vitro* that was defective in telomere elongation *in vivo*. This suggests that there may be species-specific requirements for pseudoknot functions.

INTRODUCTION

In most eukaryotes, the physical chromosome end is constituted by telomeres, tandem repeats of short G-rich sequences complexed with proteins (1). Telomerase, the telomere-specific DNA polymerase counterbalances the 'end replication problem' by synthesizing telomeric tracts (1,2). The enzyme possesses two core components, a catalytic reverse transcriptase subunit TERT (telomerase reverse transcriptase) and an RNA subunit TR (telomerase RNA) that carries the template for the replenishment of the telomeres (3). The genes encoding the two core telomerase subunits have been cloned from various eukaryotic organisms (3). The TERT subunit can be divided into three regions: the N-terminus, the RT domain and the C-terminus (4). The N- and C-terminal regions of TERT, which are not conserved among reverse transcriptases, mediate telomerase-specific functions: both are implicated in telomerase activity, processivity, multimerization, localization and telomere maintenance (4). However, binding of TERT to TR appears restricted to the N-terminus (5–9). The TR subunit varies considerably in length: 150–200 nt in ciliates, ~1300 nt in yeasts, and 400–600 nt in vertebrates (10–12). Despite a poor primary sequence conservation between ciliate telomerase RNAs, phylogenetic comparisons supported by chemical modification and protection analyses revealed that these RNAs share a common secondary structure (13–15). Similar analyses have been used to demonstrate that vertebrate telomerase RNAs also share a common secondary structure (10,16).

Interestingly, in the ciliate, vertebrate and yeast telomerase RNAs, a pseudoknot domain occupies a similar position, 3′ of the template (10,17). Mutations expected to disrupt the

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pseudoknot's putative helices in ciliate and vertebrate telomerase RNAs impair catalytic activity and compensatory mutations restore activity, supporting the existence of the pseudoknot structure (18–22).

In vivo, alteration of *Tetrahymena* pseudoknot topology leads to defects in enzyme assembly and activity (19) and *in vitro*, the disruption of the pseudoknot sequences alters the reconstitution of an active enzyme (23,24). Similarly, in vertebrates and yeasts, both the sequence and topology of the pseudoknot are critical for telomerase activity. Mutations in the human telomerase RNA (hTR) P3 pseudoknot helix (Figure 1B) significantly alter the reconstitution of a catalytically active enzyme *in vitro* (21,22). Using cells derived from the telomerase RNA (mTR) knockout mouse, Martin-Rivera *et al*. (20) demonstrated that all mutations disrupting the pseudoknot sequence and putative P3 helix structure affect *in vivo* reconstitution of telomerase activity. Similarly, disrupting the putative long-range base-pairing of the budding yeast *Kluyveromyces lactis* pseudoknot TR affects telomerase activity (25).

Telomerase, like all nucleic acid polymerases, performs nucleotide addition (type I) processivity. However, telomerase possesses a unique feature, repeat addition (type II) processivity, defined as the reiterative copying of the template to generate multiple repeats before dissociation from a single DNA substrate (26). Early experiments in which a hybrid telomerase enzyme assembled *in vivo* from *Glaucoma chattoni* telomerase RNA and *Tetrahymena thermophila* telomerase proteins implicated nontemplate RNA domains in processivity (27). Specifically, the pseudoknot has been implicated in the processivity of both the *Tetrahymena* and human enzymes. The pairing potential of the pseudoknot helices (IIIa/IIIb) as well as the entire helix III are important in conjunction with helix IV for repeat addition processivity of *Tetrahymena* telomerase (Figure 1A) (24). Mutations in the hTR P3 pseudoknot helix severely affects DNA synthesis levels and repeat addition processivity (8,22). In addition, other elements such as the P1 helix as well as nucleotides 3′ of the template (Figure 1B) in the pseudoknot/template region (hTR nucleotides 1–209) also regulate processivity (8,21). The pseudoknot/template domain (nucleotides 1–102) of the *Tetrahymena* telomerase RNA (tTR) contains a template-adjacent motif, the template recognition element (28), which may act in concert with helix IV to stimulate processivity (29). Similarly, in *Saccharomyces cerevisiae* the pseudoknot/template domain contains a stem–loop (different from the pseudoknot itself) that plays a role in template sequence usage and template boundary definition (30). Accordingly, the P1 helix of the human pseudoknot/template domain constitutes a determinant for template boundary definition (31).

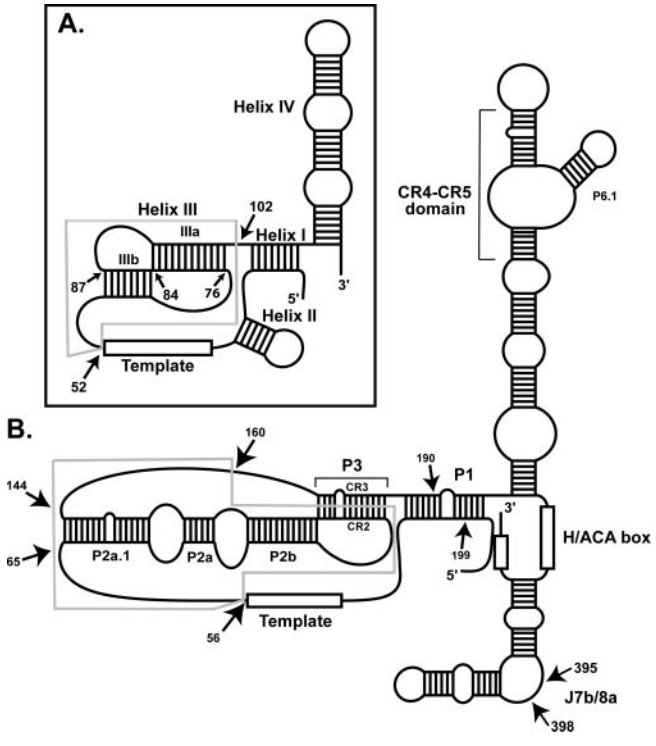

Figure 1. (**A** and **B**) Secondary structures of hTR and tTR [adapted from (10,13)]. Helices I, II, III, IIIa/IIIb and IV and the template are indicated for tTR. The template, helices P1, P2a.1, P2a, P2b, P3 (CR2–CR3), P6.1, the H/ACA box, the CR4–CR5 and J7b/8a regions are indicated for hTR. The arrows indicate the boundaries of the mutations in the tTR and hTR variants used in this study. The boxes highlight the hTR and tTR nucleotides deleted and inserted respectively, to construct the htTR chimera.

The hTR P3 pseudoknot helix has also been implicated in hTR dimerization (32). Dimerization seems to be critical for human enzyme activity, however *Tetrahymena* telomerase is active as a monomer (32,33). Although the pseudoknot and pseudoknot/template regions are directly involved in binding of TR to TERT in yeasts and human, respectively (6,8,34,35), in *Tetrahymena*, the binding of TR to TERT is not dependent on the pseudoknot *in vitro* (23).

The pseudoknot domain is also implicated in telomere maintenance. Dyskeratosis congenita (DC) is a rare multisystem syndrome characterized by defects in highly regenerative tissues, increased tumour susceptibility, premature aging, and early death due to bone marrow failure (36,37). The autosomal dominant form of the disease is characterized by mutations in hTR, some of which are located in the pseudoknot/template domain (36–39). Short telomeres are observed in autosomal dominant DC suggesting a correlation between telomere length maintenance and DC phenotypes (37). Likewise, mutations disrupting the pairing potential of *S.cerevisiae* and *K.lactis* telomerase RNA pseudoknots impair telomerase action *in vivo*, causing a marked decrease in telomere length (25,30).

Thus, not only are the structure and template-adjacent position of the pseudoknot domain conserved in the telomerase RNAs from ciliates, yeasts and vertebrates, but the pseudoknot domain also mediates similar functions in these organisms. Thus, we considered that the pseudoknot regions from different telomerase RNAs might be functionally equivalent. To test this hypothesis, we exchanged the hTR pseudoknot for the *Tetrahymena* telomerase RNA pseudoknot, creating an hTR-tTR chimera (called htTR). htTR reconstituted a weakly active, nonprocessive chimeric human telomerase enzyme *in vitro* that was defective in telomere elongation. Functional impairment could not be attributed to a lack of htTR–hTERT interactions, *in vitro* and *in vivo*, or to defects in dimerization *in vitro*.

MATERIALS AND METHODS

Constructs

phTR+1 (40) and pET28b-hTERT WT (41), pCR3.1-Flag-hTERT WT (5) and pCR3.1-Flag-hTERT 1-250, 300-600 (8) were described previously. The pET28a-tTERT plasmid was a gift from Dr T.R. Cech (Howard Hughes Medical Institute, Boulder, CO).

The method to construct phtTR was adapted from Ho *et al*. (42). Four sets of primers were designed: the primer hTR-tTR1 (5′-GGGGAAGCTTTAATACGACTCACTAT-AGGGTTGCGGAGGGTGGGCCTG-3′) which contains nucleotides (nt) 1–18 of hTR. The primer hTR-tTR2 (5′-GAAGGTTATATCAGCACTAGATTTGTTAGGGTTAGA-CAAAAAATGGCC-3′) contains nt 33–56 of hTR plus nt 52–82 of tTR. The primer hTR-tTR3 (5′-GGCCATTTTT-TGTCTAACCCTAACAAATCTAGTGCTGATATAACCT-TC-3′) is the exact complement of the primer hTR-tTR2. The primer hTR-tTR4 (5′-GCTCTAGATTACCACTTATTT-GAACCTAATTG-3′) contains nt 82–102 of hTR plus nt 159–183 of hTR. In the first PCR step (step I), 1 ng of the pGRN33 plasmid [genomic clone of hTR, (43)] was amplified by using 1 μM final of each hTR-tTR 1 and 2 primer, 1× Pfu PCR buffer (Stratagene), 0.25 mM dNTPs, 5 μl dimethyl sulfoxide (Sigma), and 2.5 U of Pfu turbo for 1 min, 94°C, 2 min, 50°C and 3 min, 72°C (30 cycles). In the second PCR step (step II), the same method as above (step I) was used to amplify the pCG1 plasmid [genomic clone of tTR (44)] with the hTR-tTR 3 and 4 primers. In the third PCR step (step III), equivalent amount (10 μl) of the PCR products from the steps I and II was mixed in the presence of hTR-tTR 1 and 4 primers, and the same method as in step I was applied to obtain a PCR product. The HindIII–XbaI treated PCR product was cloned into phTR+1 digested with the same enzymes.

phTR 1–56 and phTR 160–451 were generated by PCR using the phTR+1 plasmid with the primers hTR-tTR1 (see above) and hTR56 (5′-CCCCGGATCCGTTAGGGTTA-GACA-3′), and the primers hTR160 (5′-GGGGAAGCTT-TAATACGACTCACTATAGGGTAGAGCAAACAAAAA-ATG-3′), and hTR451 (5′-CCCCGGATCCTGCGCATGT-GTGAGCCGAGTCCTGGG-3′), respectively. BamHI–HindIII treated PCR products were cloned into pUC119 digested with the same enzymes.

phTR (phTR P1sub and phTR 395–398) and phtTR (phtTR stem IIIa, phtTR stem IIIb, phtTR P1sub and phtTR 395–398) substitution variant constructs were generated by the Quick Change Site-Directed Mutagenesis method from Stratagene. The following primers were used: PN 84–87 F (5′-GCTGATATAACCTTCACCAATTACCAACAATAAGTG-GTAATAGAGC-3′) and PN 84–87 R (5′-GCTCTATTAC-CACTTATTTGTTGGTAATTGGTGTTGGTTATATCAG-C-3′) to generate the phtTR stem IIIb plasmid in which nt 84–87 are replaced by nt 69–72; PN F (5′-GT-GCTGATATAACCTTCTGGTGAATGGTTCAAATAAGT-GGTAATCTAGAGC-3′) and PN R (5′-GCTCTAGATTAC-CACTTATTTGAACCATTCACCAGAAGGTTATATCAG-CAC-3′) to generate the phtTR stem IIIa plasmid in which nt 76–83 are replaced by nt 92–99; hTR190-QCM-F (5′-CAAACAAAAAATGTCAGCTGCTGCTGGGGUGGUGG-GGCCTCCCGGGGACCTGCGGCGGGT-3′) and hTR190-QCM-R (5′-ACCCGCCGCAGGTCCCCGGGAGGCCCCA-CCACCCCAGCAGCTGACATTTTTTGTTTG-3′) to generate the phTR P1sub and phtTR P1sub plasmids in which hTR nt 190–199 are replaced by hTR nt 27–36; hTR395-398F (5′-GGAACGGAGCGAGTCCCCGCTTTTGGCGCGATTCCC-TGAGC-3′) and hTR395-398R (5′-GCTCAGGGAATCGC-GCCAAAAGCGGGGACTCGCTCCGTTCC-3′) to generate the phTR 395–398 and phtTR 395–398 plasmids in which nt 395–398 are replaced by TTTT. The underlined sequences in the primers represent the mutations introduced. The pGEM36 plasmid, encoding hTR C_4A_2 which dictates the synthesis of T_2G_4 sequences, was described previously (40).

The pcDNA3-Hygromycin-hTR and pcDNA3-Hygromycin-htTR constructs were generated from the pcDNA3-Hygromycin plasmid (Invitrogen, gift from Dr A. Koromilas, Lady Davis Institute Montreal, Quebec, Canada). HindIII–BamHI-digested hTR and htTR from the phTR+1 and phtTR plasmids, respectively, were cloned into the pcDNA3-Hygromycin plasmid digested with the same enzymes. The identities of all the constructs were verified by restriction enzyme analysis and/or sequencing.

Cell line, transfection and cellular extract

The VA13-hTERT is a telomerase-negative ALT (alternative lengthening of telomeres) cell line which has been transfected with the human telomerase catalytic subunit, hTERT (45) (kindly provided by Dr S. Bachetti, Regina Elena Cancer Institute, Rome, Italy). VA13-hTERT cells were grown in α-MEM with 10% fetal bovine serum (FBS). Stable cell lines were obtained by transfection with the vector pcDNA3 alone, pcDNA3-hTR or pcDNA3-htTR. For the cellular extracts, cells were lysed in NP-40 lysis buffer [10 mM Tris–HCl (pH 7.5), 1mM $MgCl_2$, 1mM EGTA, 10% glycerol, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol and 1% NP-40] and incubated on ice for 10 min. The cell lysates were centrifuged at 13 226 *g* for 20 min at 4°C. The supernatant was further used for the experiments. Clonal populations were selected with 105 μg/ml hygromycin for 3–4 weeks, and were routinely subcultured at a 1:4 split ratio as they reached confluence.

RNA isolation and RT–PCR

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instruction. Reverse transcription of the RNAs was followed by PCR to detect hTR WT and htTR expressions with the following primers: 5′-TCTAACCC-TAACTGAGAAGGGCGTAG-3′ and 5′-GTTTGCTCTA-GAATGAACGGTG-3′. hTERT expression was revealed with the primers hT1, 5′-AAGTTCCTGCACTGGCTGAT-GAG-3′ and hT2, 5′-TCGTAGTTGAGCACGCTGAACAG-3′. Human GADPH was amplified with primers RT11, 5′-CGGATCAACGGATTTGGTCGTAT-3′ and RT12, 5′-TGCTAAGCAGTTGGTGGTGCAGGA-3′ as a control.

In vitro reconstitution of telomerase, TRAP and direct primer extension assays

TERT proteins were synthesized in rabbit reticulocyte lysate (RRL) by incubating 25 ng of various TERT constructs per μl of RRL in the presence of *in vitro* transcribed telomerase RNAs (10 ng/μl of RRL) and [^{35}S]methionine. Direct primer extension telomerase assay was performed as described

previously (7), except that 12.5 μM of each primer were used per reaction. The TRAP (telomeric repeat amplification protocol) assay was performed as described previously using the ACX primer (7), except for the experiments reported in Figure 2B, which were performed with the ACT-C_4A_2 primer (5′-GCGCGGCCAACCCCAACCCCAACCCCAACC-3′) where indicated.

The ladder of products generated by the RRL-reconstituted enzymes and the products corresponding to the PCR internal control (IC) were quantified using Molecular Dynamics

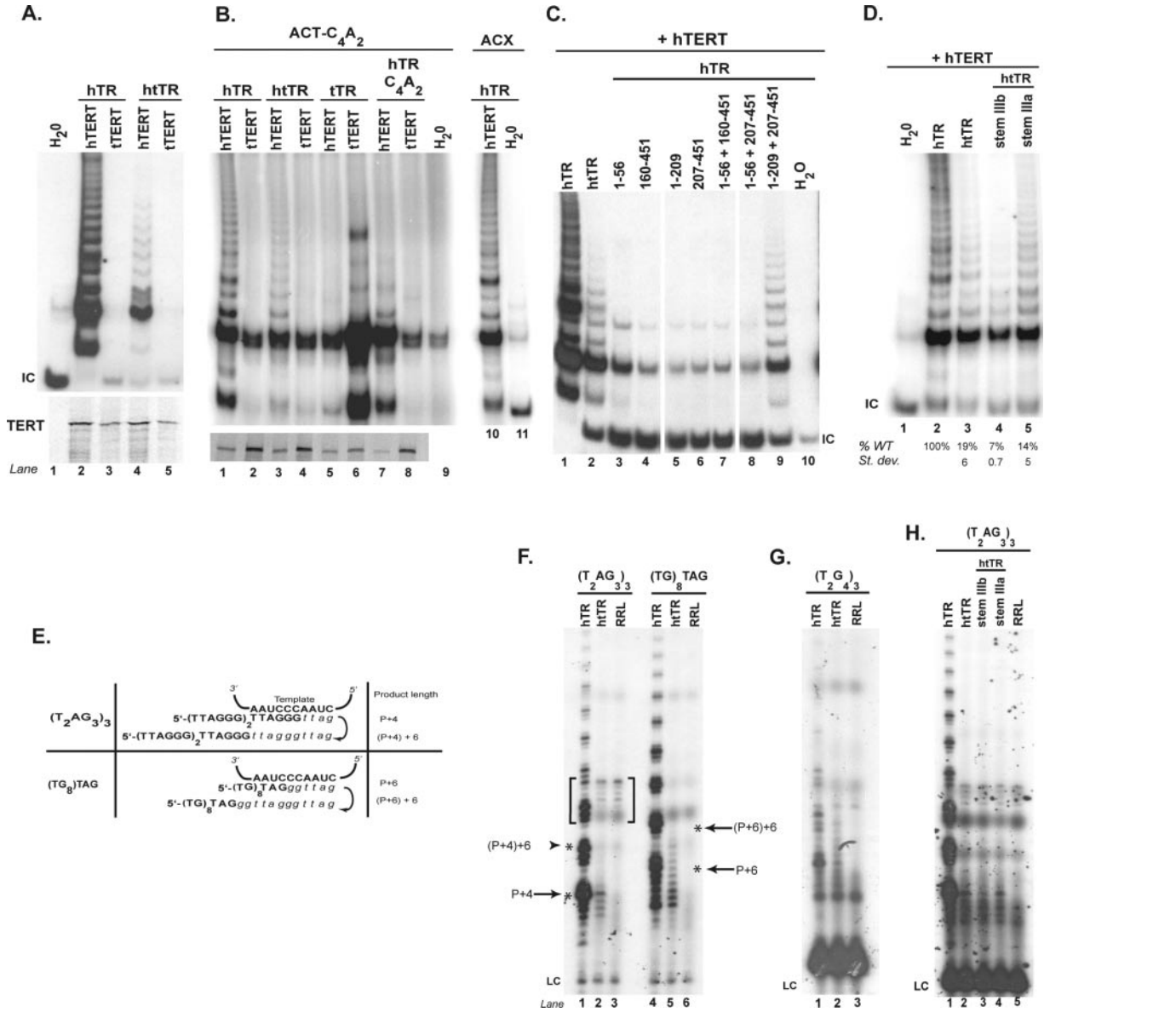

Figure 2. The chimera reconstitutes human telomerase activity upon hTERT expression in *vitro*. (**A**) Upper panel. The chimera reconstitutes telomerase activity by TRAP. Telomerase complexes were reconstituted in RRL, and telomerase activity was detected using TRAP. IC indicates the internal PCR control. Lower panel. *In vitro* synthesis of hTERT and tTERT. 1 μl of the corresponding RRL reaction was analyzed by SDS–PAGE. (**B**) Upper panel. tTR does not function with hTERT to reconstitute telomerase activity by TRAP. Telomerase complexes were reconstituted in RRL, and telomerase activity was detected by TRAP, using a modified primer complementary to the template sequence of tTR (ACT-C_4A_2) (lanes 1–9) or the ACX primer (lanes 10 and 11). Lower panel. *In vitro* synthesis of hTERT and tTERT. The corresponding RRL reaction of 1 μl was analyzed by SDS–PAGE. (**C**) hTR1-56 and hTR160-451 do not reconstitute telomerase activity as measured by TRAP. The same reconstitution method as in (A) was used. (**D**) A mutation within the tTR pseudoknot sequences in htTR significantly reduces the activity reconstituted by the chimeric enzyme. The same method as in (A) has been used. %WT indicates the average percentage of WT telomerase activity derived by quantification of the variants' telomerase activities relative to the WT telomerase activity and the standard deviation. A total of five experiments were performed. (**E**) Diagram depicting the predicted alignments of the oligonucleotides (used in F and H) with the hTR template. The arrow indicates the translocation of the primer along the template after the first round of DNA synthesis by the telomerase enzyme. P stands for primer length. (**F**) The chimeric enzyme is nonprocessive. RRL-reconstituted human telomerase catalytic activity was tested using the direct primer extension telomerase assay with the indicated 3′ end-labeled biotinylated primers. (P + 4), (P + 4) + 6, (P + 6), (P + 6) + 6 indicate the position of the products synthesized by telomerase (arrows, asterisks). (**G**) The chimeric enzyme nonprocessively elongates a *Tetrahymena*-like telomeric oligonucleotide, $(T_2G_4)_3$. The same method as in (F) was used. (**H**) The htTR stem IIIa- and stem IIIb-reconstituted enzymes are nonprocessive. The same method as in (F) was used.

Densitometer and ImageQuant software as previously described (7). The quantification of the nucleotide addition processivity of the telomerase enzymes was performed as described previously (7).

In vitro RNA binding assay

RNA binding was performed as described previously (8), except that 8.82 μg of α-Flag M2 antibody (Sigma) or 5.4 μg of anti-hTERT antibody (6) per ml were used for immunoprecipitations. The FLAG RID2 hTERT protein was immunoprecipitated with the α-Flag antibody. hTERT was immunoprecipitated with the anti-hTERT antibody (6). The quantifications were performed as described previously (6).

In vitro hTR/htTR dimerization assay

Dimerization assays were performed as described previously (32). The monomers and dimers formed by each RNA at 37°C were quantified using Molecular Dynamics Densitometer and ImageQuant software. For monomer and dimer signals in each lane, background (region directly above signals) was subtracted and the percentage of dimer for one RNA at 37°C was determined by the following formula: (corrected dimer value)/(corrected monomers values + corrected dimers values) × 100.

Q-FISH

Q-FISH assay was performed as described previously (46).

Immunoprecipitation assay, northern and western blots analyses

A total of 50 μg of extracts from two hTR-transfected, two htTR-transfected and one vector-transfected clones were immunoprecipitated with the anti-hTERT antibody (6). Final volume of 100 μl beads were divided in three aliquots: 10 μl were dedicated to a TRAP assay, 30 μl to the western blot and 60 μl to the northern blot analyses. 30 μl of beads were loaded on a SDS–PAGE. Anti-hTERT antibody of 0.3 μg/ml was used to reveal the presence of the hTERT proteins. The RNAs were extracted from the 60 μl of beads with phenol/chloroform/isoamyl. The northern blots were probed with a ^{32}P-labeled hTR cDNA directed against a common region of hTR and htTR.

RESULTS

Construction of an hTR-tTR pseudoknot chimera

To test the functional equivalence of the human and *Tetrahymena* telomerase RNA pseudoknot domains, we exchanged hTR pseudoknot sequences for tTR pseudoknot sequences while maintaining the other domains of hTR, including the P1 helix (Figure 1B). The pseudoknot/template domain of hTR (nucleotides 1–209) contains several regions: the P1 helix, the template and the pseudoknot, comprised of helices P2a.1, P2a, P2b and P3 (10) (Figure 1B). As we were interested in understanding the effect of a pseudoknot exchange on human telomerase function, we kept the hTR template (nucleotides 46–56), necessary to assay human telomerase activity; hTR nucleotides 170–200 were also maintained in the chimera because of their importance for human telomerase catalytic activity (40,47,48). Thus, hTR nucleotides 57–159 were deleted, including helices P2a.1, P2a, P2b and part of P3, and replaced by tTR nucleotides 52–102, including pseudoknot helices IIIa and IIIb (Figures 1A and B). The resulting chimera, called htTR, comprises nucleotides 1–56 of hTR, 52–102 of tTR and 160–451 of hTR.

Expression of the chimera and hTERT *in vitro* reconstitutes telomerase activity as measured by TRAP

To test if the introduction of the tTR pseudoknot in the hTR backbone would generate an *in vitro* catalytically functional enzyme, we first analysed the activity of the chimeric enzyme by TRAP. We reconstituted the chimeric telomerase enzyme by expressing hTERT in the presence of htTR using an *in vitro* transcription and translation RRL system. Since the chimera contains *Tetrahymena* pseudoknot sequences, we also tested whether htTR could reconstitute telomerase activity upon expression of the *Tetrahymena* catalytic subunit tTERT.

Telomerase reconstituted with htTR exhibited decreased activity compared to the enzyme reconstituted with hTR (Figure 2A, lanes 2 and 4). No reconstitution of telomerase activity was detected with hTR and htTR upon expression of tTERT (upper and lower panels of Figure 2A, lanes 3 and 5), suggesting that tTERT cannot function with hTR, or the tTR region in htTR, to reconstitute telomerase activity. Thus, the reconstituted chimeric human enzyme was partially active by TRAP *in vitro*.

To determine if tTR could reconstitute activity with hTERT, we analyzed telomerase activity by TRAP using a modified primer complementary to the tTR template sequence (ACT-C_4A_2) (Figure 2B). An hTR mutant, hTR C_4A_2, reprogrammed to direct the synthesis of T_2G_4 repeats by human telomerase was used as a positive control (40,43). Enzymes reconstituted with hTERT and hTR or htTR (upper and lower panels of Figure 2B, lanes 1 and 3), but not with tTR (Figure 2B, lane 5) generated elongation products. However, as previously shown with the ACX primer (Figure 2A), no telomerase activity was detected with enzymes reconstituted with tTERT and hTR or htTR (upper and lower panels of Figure 2B, lanes 2 and 4). Some elongation products were detected for enzymes reconstituted with tTERT and tTR (upper and lower panels of Figure 2B, lane 6), and with hTERT and hTR C_4A_2 (upper and lower panels of Figure 2B, lane 7). Similar TRAP elongation products generated by telomerase reconstituted with hTR C_4A_2 have been reported previously (40,43).

hTR1-56 and hTR160-451 do not reconstitute telomerase activity *in vitro* as measured by TRAP upon hTERT expression

A functional human telomerase can be reconstituted *in vitro* by expressing hTERT, and combining *in trans* two independently inactive hTR fragments, the pseudoknot/template and the CR4–CR5 domains (47–49). Thus, we asked if the hTR sequences remaining in the chimera could be sufficient to mediate its activity. We constructed the hTR variants 1–56 and 160–451, which correspond to the hTR regions present in the chimera, and tested their capacity to generate telomerase activity *in trans* upon hTERT expression. The hTR fragments 1–209 and 207–451 were used as a positive control, since in

concert with hTERT they reconstitute partial telomerase activity *in vitro* (49).

The hTR fragments 1–209 and 207–451 did not independently reconstitute telomerase activity (Figure 2C, lanes 5 and 6), but reconstituted partial telomerase activity when added *in trans* (Figure 2C, lane 9) (49). On the contrary, hTR 1–56 and hTR 160–451 did not independently reconstitute telomerase activity in the presence of hTERT (Figure 2C, lanes 3 and 4) or when mixed *in trans* with hTERT (Figure 2C, lane 7). This result suggests that hTR nucleotides 1–56 and 160–451 may not be sufficient to reconstitute telomerase activity in the context of the chimera, and that tTR sequences 52–102 may play a role in the function of the chimeric RNA.

Mutation of tTR pseudoknot sequences in htTR impairs reconstitution of telomerase activity as measured by TRAP

Disrupting the putative base-pairing of the pseudoknot structure in ciliate and human telomerase RNAs results in impairment of the catalytic activity of the telomerase complex, highlighting the importance of the formation of this secondary structure for the enzyme's function (18–22). To test if the chimeric enzyme activity was dependent on the tTR pseudoknot sequences and potentially structures, we created substitution mutations within the sequences implicated in the formation of the tTR pseudoknot, stem IIIa and stem IIIb (Figure 1A). Specifically, tTR stem IIIb nucleotides 84–87 were replaced by nucleotides 69–72 (htTR stem IIIb) and tTR stem IIIa nucleotides 76–83 were replaced by nucleotides 92–99 (htTR stem IIIa) (Figure 1A).

Telomerase reconstituted with htTR stem IIIb and htTR stem IIIa exhibited 7 and 14%, respectively, of the activity reconstituted with hTR, compared to 19% for htTR (Figure 2D, lanes 2–5). Thus, reconstitution of activity by htTR was significantly impaired by the stem IIIb substitution, indicating that tTR pseudoknot sequences and possibly structures contribute to the activity of the chimeric enzyme

The chimeric enzyme is nonprocessive *in vitro*

The pseudoknot/template region of hTR constitutes a domain that regulates the processivity of human telomerase (8,21). When hTR sequences are substituted for mTR residues in the pseudoknot/template domain, the reconstituted telomerase enzyme demonstrates defects in processivity (21). Similarly, deletion and substitution mutations between hTR nucleotides 65 and 208 results in a complete loss of repeat addition processivity (8). In the same way, the *Tetrahymena* telomerase RNA pseudoknot has been implicated in processivity (24). To characterize the repeat addition processivity of the chimeric enzyme, we performed a direct primer extension assay.

The chimeric enzyme synthesized less DNA than the WT enzyme onto the telomeric primer $(T_2AG_3)_3$ (Figure 2F, lanes 1 and 2). Furthermore, the chimeric enzyme seemed to be defective in repeat addition processivity, since it was unable to synthesize more than one telomeric repeat (Figure 2F, lane 2). The observed longer products (Figure 2F, brackets) were non-specific, as they were also generated in control RRL reactions (Figure 2F, lane 3) and were absent in reactions performed using a different primer substrate (Figure 2F, lanes 4–6).

To test the specificity and reproducibility of the results, we used another primer, $(TG)_8TAG$ (50). This primer aligns more distally from the 5′ boundary of the RNA template than the primer $(T_2AG_3)_3$ (Figure 2E) (50). As expected, the DNA product profile of the WT enzyme shifted upwards compared to the elongation product pattern with $(T_2AG_3)_3$ (Figure 2F compare lanes 1 and 4), indicating that 6 nt rather than 4 nt were added to the DNA substrates before reaching the 5′ template boundary (50). As with the $(T_2AG_3)_3$ primer, the chimeric enzyme exhibited an extensive loss of repeat addition processivity compared to the WT enzyme (Figure 2F, lanes 4 and 5). In addition, the chimeric enzyme was also defective in nucleotide addition processivity, specifically at position P + 2 (T in the sequence TTAGGG) where processivity was reduced by 32% compared to the wild-type enzyme. In the event that sequences in the tTR pseudoknot might contribute to primer interaction or primer utilization, we tested the $(T_2G_4)_3$ primer (Figure 2G). Though both WT and chimeric enzymes were able to elongate the $(T_2G_4)_3$ primer, the chimeric enzyme exhibited an extensive loss of repeat addition processivity compared to the WT enzyme, as with the other previously tested primers (Figure 2G, lanes 1 and 2). Therefore we concluded that the RRL-reconstituted chimeric enzyme exhibited defects in repeat addition processivity and levels of DNA synthesis.

Telomerase reconstituted with htTR stem IIIa or IIIb and assayed by the direct method generated activity levels similar to those of telomerase reconstituted with htTR (Figure 2H). Varying levels of long, undetectable elongation products generated by htTR-, htTR stem IIIa- and htTR stem IIIb-reconstituted enzymes may be detectable by the more sensitive PCR-based TRAP assay. Indeed, previous studies similarly reported that enzymes with major defects in repeat addition processivity can generate TRAP-detectable products (7).

The chimeric RNA interacts with hTERT *in vitro*

The pseudoknot/template and CR4–CR5 domains are the two hTR regions required for hTERT binding (47,49). Since the human pseudoknot nucleotides 57–159 are absent from the chimera (Figure 1B), we tested the chimeric RNA for its ability to bind hTERT. We used non-radiolabeled competitor RNAs (hTR, positive control, and htTR) in the presence of ^{35}S-labeled hTERT and ^{32}P-labeled hTR reconstituted in RRL, in a co-immunoprecipitation assay with an anti-hTERT antibody. The binding of hTR to hTERT in the absence of non-radiolabeled competitor RNA was set as the maximum binding [100%, Figure 3A (lane 2) and B]. Increasing concentrations of non-radiolabeled hTR efficiently inhibited the interaction of ^{32}P-labeled hTR with hTERT (Figure 3A, lanes 3–5), reducing its binding to 26, 10 and 4%, respectively (Figure 3B). Visually, the non-radiolabeled chimeric RNA did not seem to inhibit the formation of the ^{35}S-labeled hTERT/^{32}P-labeled hTR complex as efficiently as non-radiolabeled hTR (Figure 3A, lanes 6–8), especially at the lowest concentrations, reducing the binding only to 51 and 15% (Figure 3B). Nevertheless, quantification of four independent experiments demonstrated that the differences observed in individual experiments were not statistically significant ($P > 0.05$) (Figure 3B). We did not observe any significant defects in the binding of htTR to the hTERT RNA interaction domain

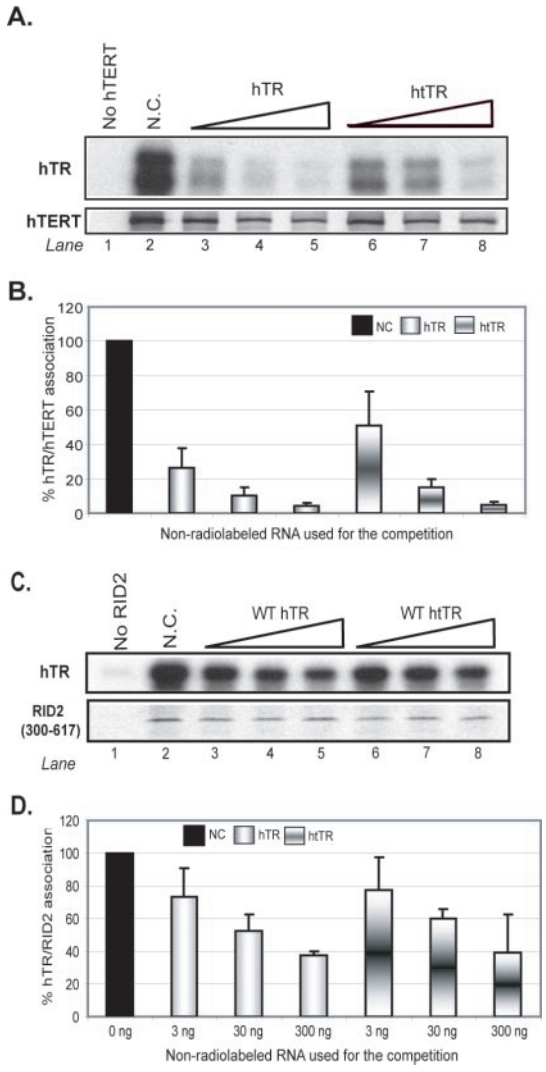

Figure 3. The chimera interacts with hTERT *in vitro*. (**A**) ^{35}S-labeled hTERT was synthesized in RRL in the presence of equal amounts of ^{32}P-labeled hTR and increasing amounts of unlabeled competitor RNAs, hTR or htTR (3, 30 and 300 ng). The formed ribonucleoprotein complexes were immunoprecipitated with an anti-hTERT antibody and visualized by SDS–PAGE. No competition (NC) (**B**) Quantification of the association between hTR and hTERT in the presence of competitor RNAs, expressed relative to the NC control. At least three independent experiments were performed. (**C**). The chimera interacts with hTERT RID2. The same method as in (A) was used. (**D**) Quantification of the association between hTR and hTERT RID2 in the presence of competitor RNAs.

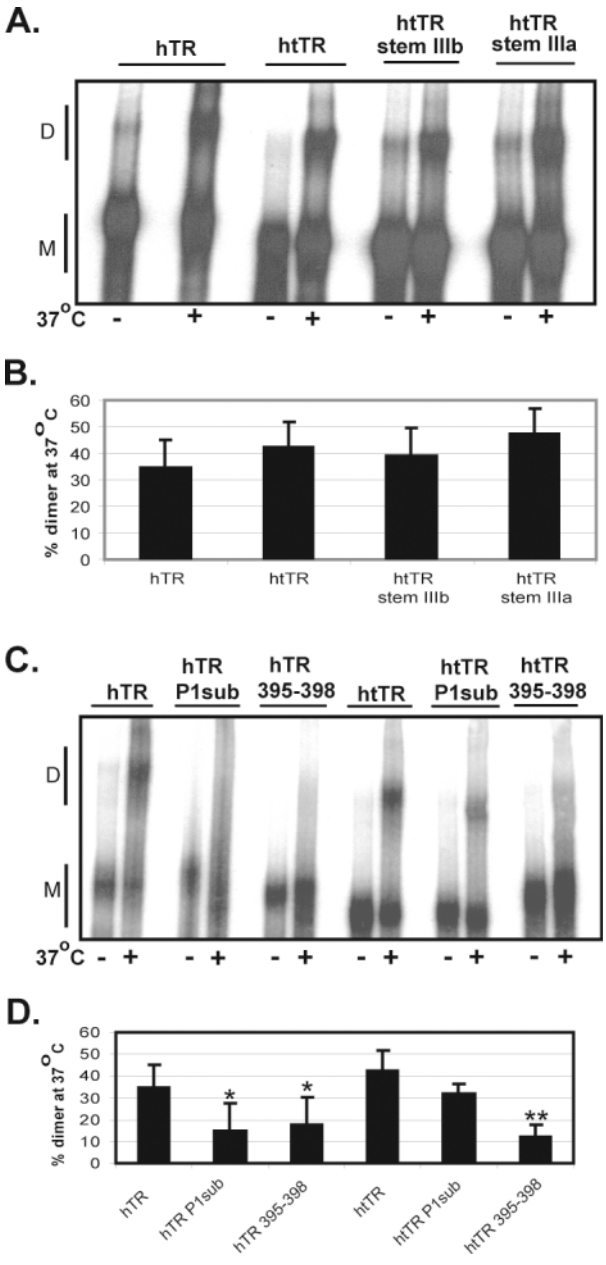

Figure 4. (**A** and **B**) The chimera dimerizes and alterations of the stem IIIa and stem IIIb in the *Tetrahymena* pseudoknot structure do not inhibit its dimerization. Representative results of dimerization for htTR mutants are shown. M and D correspond to monomer and dimer positions, respectively. (B) Quantification of the dimerization results for the htTR mutants. A minimum of three independent experiments was performed for each RNA. (**C** and **D**) Mutation in the J7b/8a region of the chimera affected its dimerization. (C) Representative results of dimerization for htTR and hTR mutants are shown. (D) Quantification of the dimerization values for the hTR and htTR mutants. Average dimerization values did significantly differ from hTR (one asterisk) or the chimera (two asterisks) in a Student's *t*-test.

2 (RID2), a high-affinity binding site reported previously to bind preferentially to the P6.1 helix of the hTR CR4–CR5 domain (6,51) (Figures 1, 3C and D). These results suggest that the CR4–CR5 domain structures are sufficiently maintained in htTR to allow association with hTERT. We concluded that the presence of the tTR nucleotides 52–102 and the absence of hTR nucleotides 57–159 in htTR did not abolish the binding of the chimeric RNA to hTERT.

The chimeric telomerase RNA dimerizes

The hTR P3 helix (CR2–CR3) is a determinant of hTR dimerization (Figure 1) (32). Since the chimera is missing the CR2 region required to form the P3 helix, we investigated the capacity of the chimera to dimerize *in vitro*. Despite the lack of the CR2 region (32), the chimera reproducibly dimerized as efficiently as hTR *in vitro* (Figure 4A and B).

We subsequently hypothesized that other regions of the chimera might mediate its dimerization. Though tTR does not dimerize (33) (data not shown), we first asked if the

tTR region present in the chimera could mediate dimerization in the context of the hTR backbone. We tested htTR stem IIIa and stem IIIb (Figures 1A and 2D) in dimerization assays. The chimeric RNAs carrying mutations in stem IIIa or stem IIIb dimerized as efficiently as hTR and htTR (Figure 4A and quantification in B). Thus, stem IIIa and stem IIIb sequences in the chimera do not appear to regulate dimerization and other regions of the hTR backbone might be responsible for its dimerization.

We recently demonstrated dimerization defects for a P1 helix hTR mutant, hTR 190, containing substitution of nucleotides 190–199 (8). Also, a single molecule fluorescence coincidence method, which allows the direct observation of hTR multimerization in solution, demonstrated that the J7b/8a single-stranded region forms a kissing loop between two hTR molecules (52). Nevertheless, because hTR 190 contains an additional small deletion of nucleotides 200 and 201 (8) and because the implication of the J7b/8a region in hTR dimerization has not been tested with the same method used to demonstrate the importance of the P3 helix, we engineered independent substitution mutations in the P1 helix and J7b/8a regions of hTR and htTR. The mutants hTR P1sub and htTR P1sub were generated by substituting hTR nucleotides 190–199 with nucleotides 27–36 and the hTR 395–398 and htTR 395–398 mutants by replacing hTR nucleotides 395–398 by a stretch of four thymidines.

Both mutations in the P1 helix (hTR P1sub) as well as in the J7b/8a region (hTR 395–398) affected hTR dimerization (Figure 4C), leading to at least a 1.75-fold significant reduction in dimerization compared to hTR (Figure 4D), suggesting that the P3 helix may not constitute the only determinant of hTR dimerization and that the P1 and the J7b/8a regions are also implicated. The situation was different for the chimera, where the P1 helix mutation seemed to affect its dimerization only partially (Figure 4C). Statistical analysis of more than three independent experiments revealed that the differences in dimerization observed between hTR, htTR and htTR P1sub were not significant (Figure 4D). Only the mutation in the J7b/8a region of the chimera significantly altered its dimerization (Figure 4C), reducing it by 3.5-fold compared to htTR (Figure 4D). We concluded that in the context of the chimera, the J7b/8a region of the hTR backbone appears to be the main regulator of dimerization.

The chimeric enzyme is defective in telomere elongation

The integrity of the pseudoknot domain influences the capacity of both the *Tetrahymena* and vertebrate enzymes to function *in vivo* (19,20,36). To investigate the effect of the chimeric RNA on the telomerase activity and assembly of the enzyme *in vivo*, we introduced hTR and htTR in an hTR-negative ALT cell line stably transfected with hTERT (VA13-hTERT) (45). ALT cells are typically characterized by extremely long (up to 50 kb) and heterogeneous telomeres, including chromosome ends without telomeric signal (46,53). First, we assessed the ability of the chimeric enzyme to function *in vivo* by measuring telomerase activity of whole-cell extracts by TRAP assay. hTR-transfected but not vector-transfected clones reconstituted telomerase activity (Figure 5A, lanes 3, 4 and 7). Contrary to RRL-reconstituted chimeric enzyme, the VA13-hTERT clones transfected with the chimera did not reconstitute telomerase activity (Figure 5A, lanes 5 and 6). The absence of telomerase activity could not be explained by a lack of expression of the chimeric RNA and/or hTERT in the htTR-transfected clones (Figure 5B, lanes 3 and 4 and C, lanes 1 and 2). We also performed experiments suggesting the unlikelihood that an insufficient concentration of cell extract or an inhibition of the PCR step in the TRAP assay was responsible for the lack of telomerase activity of the htTR-expressing clones (data not shown).

We then examined the effect of the reconstituted enzymes on telomere length at the single cell level by Q-FISH analysis. Telomerase reconstituted in the telomerase-negative VA13 ALT cell line by the expression of hTR and hTERT elongates the shortest telomeres, which results in a marked decrease in the number of chromosome ends without signal (i.e. naked extremities) (45,46,54,55). Indeed, in the hTR-expressing clones, the percentage of naked extremities was significantly reduced compared to the vector clone (Figure 5D). However, in the htTR-expressing clones, no significant differences in the percentage of naked extremities compared to the vector clone were detected (Figure 5D), suggesting that the reconstituted chimeric enzyme does not function to elongate the shortest telomeres. Hence, based on the absence of telomerase activity in whole-cell extracts from htTR-expressing clones, and the inability of the htTR-expressing clones to elongate the shortest telomeres, it appears that the chimeric enzyme's *in vivo* functions are defective.

The chimeric RNA interacts with hTERT *in vivo*

As the chimeric enzyme is functionally impaired *in vivo*, we asked if the defects could be due to an absence of htTR–hTERT interaction. To test this hypothesis, we immunopurified the reconstituted wild-type and chimeric complexes with an antibody directed against hTERT. We then analyzed the immunopurified complexes for their activity, the expression of the hTERT protein and the presence of the different RNAs by TRAP assay, western and northern analyses, respectively. The western and northern analyses revealed that the chimeric RNA or hTR were present in the immunopurified complex together with the hTERT protein (Figure 5F). However, the telomerase activity levels detected for the immunopurified complexes paralleled the telomerase activity levels reported for the crude whole-cell extracts: the chimeric complex did not reconstitute telomerase activity whereas the wild-type complex did (compare Figure 5A with E). We concluded that the loss of *in vivo* activity does not seem to be due to a lack of htTR–hTERT interaction.

DISCUSSION

Based on our hypothesis that the human and *Tetrahymena* pseudoknot might be functionally interchangeable, we constructed an hTR-tTR chimera, called htTR, which contains the tTR pseudoknot (nucleotides 52–102) flanked by hTR nucleotides 1–56 and 160–451. htTR reconstituted a weakly active, nonprocessive chimeric human telomerase enzyme *in vitro*, defective in telomere elongation *in vivo*. Functional impairment could not be attributed to a complete loss of htTR–hTERT interaction, *in vitro* or *in vivo*, or to defects in dimerization *in vitro*.

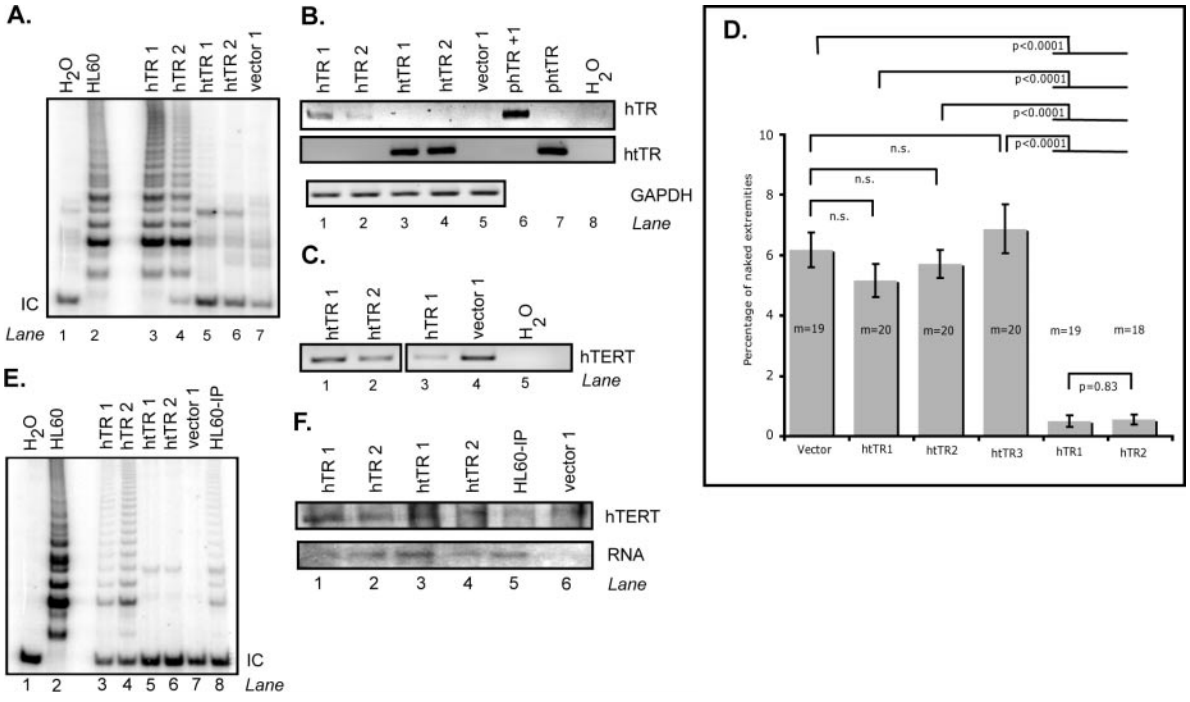

Figure 5. The chimera is defective in telomere elongation. (**A**) Telomerase activity of the VA13-hTERT clones. A total of 1 μg of whole-cell extracts was assayed for telomerase activity by TRAP. As a positive control, 1 μg of whole-cell extract from telomerase-positive HL60 was tested for telomerase activity by TRAP, IC, internal PCR control. (**B**) hTR and htTR expression in the VA13-hTERT clones. cDNA from VA13-hTERT clones transfected with pcDNA3-hTR, pcDNA3-htTR or pcDNA3 were analyzed by PCR for the expression of hTR or htTR. The plasmids phTR + 1 and phtTR were used as positive controls for the PCR and GADPH as an IC for the integrity of the cDNAs. (**C**) hTERT expression in the VA13-hTERT clones. cDNA from VA13-hTERT clones transfected with hTR, htTR or the vector were analyzed for the expression of hTERT by PCR. (**D**) Quantification of the naked extremities in VA13-hTERT clones by Q-FISH. A minimum of 15 metaphases were prepared for each clone (as indicated by the number on the bar of the graph). n.s., non statistically significant; m, number of metaphases prepared. The results were expressed as a ratio of naked extremities over the total extremities measured by Q-FISH in one vector clone, two clones expressing hTR and in three clones expressing htTR. Average values were analyzed by a Student's *t*-test, and *P*-values from comparison between different clones are indicated. (**E** and **F**) The chimera assembles with hTERT *in vivo* but is not active by TRAP. Telomerase was immunoprecipitated from whole-cell extracts made from hTR-transfected, htTR-transfected or vector-transfected clones and the telomerase-positive human cell line, HL60, using an anti-hTERT antibody (E) 10% of the resulting beads were used to perform a TRAP assay on the immunopurified complex. Thirty percent of the beads were used to reveal the presence of the hTERT protein by western blot analysis with the same antibody used for the immunopurification (F, top panel). Sixty percent of the beads were used to reveal the presence of hTR and htTR in the immunopurified complex by northern blot with a probe directed against a common region of the two RNAs (F, bottom panel).

The chimeric RNA reconstituted a weakly active nonprocessive human telomerase enzyme *in vitro*

Our results indicate that the inserted tTR pseudoknot sequences may play a role in mediating the activity of the chimeric enzyme. First, htTR reconstituted 19% of the activity (measured by TRAP) reconstituted by hTR. Second, mixing hTR 1–56 and hTR 160–451 *in trans* upon hTERT expression did not reconstitute telomerase activity. Importantly, disrupting tTR pseudoknot sequences and potentially structures significantly impaired the reconstitution of an active chimeric enzyme.

Furthermore, we showed using the non PCR-based, direct primer extension assay that htTR reconstituted a nonprocessive enzyme that is defective in DNA and multiple repeat syntheses. Low undetectable levels (by the primer extension assay) of longer elongation products could be sufficient for detection by the more sensitive PCR-based TRAP assay. Indeed, previous studies similarly reported that enzymes with major defects in repeat addition processivity can generate TRAP-detectable products (7).

Both *Tetrahymena* and human pseudoknot domains are crucial for repeat addition processivity (8,24). Our results suggest that in the context of the hTR backbone, tTR pseudoknot sequences cannot mediate repeat addition processivity. In addition, disruption of the P3 helix in htTR may contribute to the impaired repeat addition of the chimeric enzyme. The *Tetrahymena* tTR helix IV cooperates with the pseudoknot's stem III to regulate repeat addition processivity, and to stimulate processivity *in trans* (24,29). Furthermore, tTR stem IV has been proposed as the functional analog of the human CR4–CR5, or the P6.1 helix (29). Thus, in the chimera, if the P6.1 helix stimulated and/or cooperated with the tTR pseudoknot sequences, this event was insufficient for the chimera to reconstitute repeat addition processivity. Additionally, though the tTR helix IV may possess some functional similarities to hTR CR4/CR5, unlike CR4/CR5, helix IV is not involved in high-affinity interactions with TERT. The interaction between hTR P6.1 helix and hTERT is essential for DNA synthesis (8). Since the chimeric enzyme synthesized reduced levels of DNA compared to the wild-type enzyme, the P6.1 helix–hTERT interaction may not be sufficient for DNA synthesis. Alternatively, the long-range interaction between the P6.1 helix and the template (56) could be impaired in the chimera due to the presence of tTR pseudoknot sequences.

The *Tetrahymena* and human pseudoknot domains adopt alternate structures (14,16,57–59). Recently, two studies

provide evidence that support a stable hTR pseudoknot structure (59,60). Stable triple helix formation strongly correlates with telomerase activity (59). It is possible that the triple helix does not form in the chimeric RNA, and may explain its defects. The proper folding of the *Tetrahymena* pseudoknot has also been reported to be TERT-dependent *in vitro* and *in vivo* (19,61). It has been proposed that the conformational transition of the pseudoknot may help to dissociate the RNA–DNA duplex before the translocation of the enzyme to generate another repeat (24,26). Thus, it is possible that the tTR pseudoknot in the chimera is unable to perform this transition, perhaps because it cannot interact with regions specific to hTERT, therefore rendering the enzyme nonprocessive.

The chimera binds hTERT

Mutations scattered throughout the pseudoknot/template domain, including in the P1 helix and near the template, do not detectably affect the binding of hTR to hTERT (22). Similarly, the chimera was able to interact with hTERT. The binding of hTR to the N-terminal hTERT RNA interaction domain 1 (RID1) has been mapped to the pseudoknot/template domain (hTR 1–209) (7,8). As RID1 is a weak affinity hTR binding site, defective interactions that might be predicted between hTERT RID1 and the altered pseudoknot/template domain in the chimeric RNA might be difficult to detect in the context of full-length hTERT and RNA, in which the high-affinity hTERT–hTR interactions mediated by RID2 and hTR CR4–CR5 are presumably intact. Alternatively, the introduced *Tetrahymena* sequences might be contributing to hTERT binding, or the hTR sequences important for binding to RID1 may map to nucleotides within 1–56 or 160–209, that are not changed in the chimera and that have not been previously altered in published studies (16,22). A TLC1–hTR chimera, called *tlc1-96*, in which the *S.cerevisiae* pseudoknot was replaced by hTR pseudoknot nucleotides 90–183, has recently been shown to demonstrate a 50-fold reduction in its binding to the catalytic subunit Est2p compared to TLC1 (35). These results and our data suggest that pseudoknot sequences or structures may be less critical for hTR interactions with hTERT than TLC1 association with Est2p.

The chimera dimerizes

The P3 helix of the human pseudoknot/template domain was first implicated in the dimerization of the human telomerase RNA (32). More recently, the involvement of the P1 helix and the J7b/8a loop in dimerization has been reported (8,52). Mutations in either region reduced hTR dimerization to levels reported for hTR P3 helix mutants, suggesting that the P1 helix and J7b/8a are as important as the P3 helix in regulating hTR dimerization. In the absence of the P3 helix in the chimera, the J7b/8a region, but not the P1 helix appeared to be important for htTR dimerization, suggesting that the P3 and P1 helices may not play a crucial role in htTR dimerization. We speculate that the presence of the tTR pseudoknot sequences may alter the normal folding of the pseudoknot/template region and impair the formation of hTR dimers via the P3 and P1 helices.

The role of hTR dimerization in telomerase function is still unclear. Our results with the chimera support the previously reported evidence that hTR dimerization is not sufficient for repeat addition processivity (8). One could also envision that the dimerization mediated by different hTR regions is not functionally equivalent. For instance, dimerization via the P3 helix, which is absent in the chimera could be the most critical for repeat addition processivity. Interestingly, the P1 helix, though it mediates dimerization, is not essential for repeat addition processivity (8). Clearly, more studies are needed in order to clarify the role of hTR dimerization in human telomerase function.

The chimeric enzyme is defective in telomere elongation

Mutations in the pseudoknot/template domains of yeast and human telomerase RNAs can impair the ability of telomerase to properly maintain telomere length. (35–37,39). Yeast expressing telomerase reconstituted with a TLC1–hTR chimeric RNA (*tlc1-96*) containing human pseudoknot sequences are also defective in the maintenance of wild-type telomere length (35). However, yeast expressing a TLC1–*Oxytricha nova* chimeric RNA maintain almost wild-type length telomeres (35).

As varying levels of mouse telomerase activity can be reconstituted by the *in vivo* assembly of mouse telomerase with pseudoknot mutant mTRs (20), and because the chimeric enzyme was partially active *in vitro*, we considered that the chimeric enzyme might be partially functional at the telomeres. Instead, no telomerase activity was detectable from whole-cell extract made from telomerase-negative cells expressing hTERT and htTR, and the chimeric enzyme was defective in telomere elongation. At the present time, we cannot dismiss the possibility that the chimeric enzyme is not recruited to the telomeres. However, the lack of activity is not due to an inability of the two components to interact since the chimera could be co-immunoprecipitated with hTERT from the whole-cell extracts. Though htTR and hTERT can interact in the cell extracts, the functional assembly of the chimeric enzyme in a cell could differ from the functional assembly in the cell-free RRL system. It is also likely that the chimeric enzyme's defects in repeat addition processivity and in DNA synthesis might be sufficient to prevent proper function at the telomeres. The defect of the chimera, which contains a large alteration of its pseudoknot region, to elongate the shortest telomeres was more pronounced that the defect in telomere elongation recently reported for a mutant hTR (hTR-DC) carrying the GC to AG double substitution in the pseudoknot nucleotides 107–108 (62). Interestingly, telomerase reconstituted with hTR-DC, which is mutated in autosomal DC, is defective in DNA synthesis *in vitro*, but exhibits almost wild-type levels of repeat addition processivity (8).

Though telomerases from ciliates, yeasts and vertebrates share common features, some distinct characteristics include, but are not limited to, differences in enzyme repeat addition processivity and telomere length. The exchange of sequences and structures between telomerases from different species such as reported here and previously (21,27,35) will continue to provide insights regarding the distinct mechanisms that mediate species-specific differences in enzyme function.

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REFERENCES

1. Smogorzewska,A. and de Lange,T. (2004) Regulation of telomerase by telomeric proteins. *Annu. Rev. Biochem.*, **73**, 177–208.
2. Chakhparonian,M. and Wellinger,R.J. (2003) Telomere maintenance and DNA replication: how closely are these two connected? *Trends Genet.*, **19**, 439–446.
3. Harrington,L. (2003) Biochemical aspects of telomerase function. *Cancer Lett.*, **194**, 139–154.
4. Kelleher,C., Teixeira,M.T., Forstemann,K. and Lingner,J. (2002) Telomerase: biochemical considerations for enzyme and substrate. *Trends Biochem. Sci.*, **27**, 572–579.
5. Beattie,T.L., Zhou,W., Robinson,M.O. and Harrington,L. (2000) Polymerization defects within human telomerase are distinct from telomerase RNA and TEP1 binding. *Mol. Biol. Cell*, **11**, 3329–3340.
6. Moriarty,T.J., Huard,S., Dupuis,S. and Autexier,C. (2002) Functional multimerization of human telomerase requires an RNA interaction domain in the N terminus of the catalytic subunit. *Mol. Cell. Biol.*, **22**, 1253–1265.
7. Huard,S., Moriarty,T.J. and Autexier,C. (2003) The C terminus of the human telomerase reverse transcriptase is a determinant of enzyme processivity. *Nucleic Acids Res.*, **31**, 4059–4070.
8. Moriarty,T.J., Marie-Egyptienne,D.T. and Autexier,C. (2004) Functional organization of repeat addition processivity and DNA synthesis determinants in the human telomerase multimer. *Mol. Cell. Biol.*, **24**, 3720–3733.
9. Lai,C.K., Mitchell,J.R. and Collins,K. (2001) RNA binding domain of telomerase reverse transcriptase. *Mol. Cell. Biol.*, **21**, 990–1000.
10. Chen,J.-L., Blasco,M.A. and Greider,C.W. (2000) Secondary structure of vertebrate telomerase RNA. *Cell*, **100**, 503–514.
11. Collins,K. (1999) Ciliate telomerase biochemistry. *Annu. Rev. Biochem.*, **68**, 187–218.
12. Singer,M.S. and Gottschling,D.E. (1994) *TLC1*: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science*, **266**, 404–409.
13. Romero,D.P. and Blackburn,E.H. (1991) A conserved secondary structure for telomerase RNA. *Cell*, **67**, 343–353.
14. Bhattacharyya,A. and Blackburn,E.H. (1994) Architecture of telomerase RNA. *EMBO J.*, **13**, 5721–5731.
15. Zaug,A.J. and Cech,T.R. (1995) Analysis of the structure of *Tetrahymena* nuclear RNAs *in vivo*: telomerase RNA, self-splicing rRNA and U2 snRNA. *RNA*, **1**, 363–374.
16. Antal,M., Boros,E., Solymosy,F. and Kiss,T. (2002) Analysis of the structure of human telomerase RNA *in vivo*. *Nucleic Acids Res.*, **30**, 912–920.
17. Chen,J.L. and Greider,C.W. (2004) An emerging consensus for telomerase RNA structure. *Proc. Natl Acad. Sci. USA*, **101**, 14683–14684.
18. Autexier,C. and Greider,C.W. (1998) Mutational analysis of the *Tetrahymena* telomerase RNA: identification of residues affecting telomerase activity *in vitro*. *Nucleic Acids Res.*, **26**, 787–795.
19. Gilley,D. and Blackburn,E.H. (1999) The telomerase RNA pseudoknot is critical for the stable assembly of a catalytically active ribonucleoprotein. *Proc. Natl Acad. Sci. USA*, **96**, 6621–6625.
20. Martin-Rivera,L. and Blasco,M. (2001) Identification of functional domains and dominant negative mutations in vertebrate telomerase RNA using an *in vivo* reconstitution system. *J. Biol. Chem.*, **276**, 5856–5865.
21. Chen,J.L. and Greider,C.W. (2003) Determinants in mammalian telomerase RNA that mediate enzyme processivity and cross-species incompatibility. *EMBO J.*, **22**, 304–314.
22. Ly,H., Blackburn,E.H. and Parslow,T.G. (2003) Comprehensive structure-function analysis of the core domain of human telomerase RNA. *Mol. Cell. Biol.*, **23**, 6849–6856.
23. Licht,J.D. and Collins,K. (1999) Telomerase RNA function in recombinant *Tetrahymena* telomerase. *Genes Dev.*, **13**, 1116–1125.
24. Lai,C.K., Miller,M.C. and Collins,K. (2003) Roles for RNA in telomerase nucleotide and repeat addition processivity. *Mol. Cell*, **11**, 1673–1683.
25. Tzfati,Y., Knight,Z., Roy,J. and Blackburn,E.H. (2003) A novel pseudoknot element is essential for the action of a yeast telomerase. *Genes Dev.*, **17**, 1779–1788.
26. Lue,N.F. (2004) Adding to the ends: what makes telomerase processive and how important is it? *Bioessays*, **26**, 955–962.
27. Bhattacharyya,A. and Blackburn,E.H. (1997) A functional telomerase RNA swap *in vivo* reveals the importance of nontemplate RNA domains. *Proc. Natl Acad. Sci. USA*, **94**, 2823–2827.
28. Miller,M.C. and Collins,K. (2002) Telomerase recognizes its template by using an adjacent RNA motif. *Proc. Natl Acad. Sci. USA*, **99**, 6585–6590.
29. Mason,D.X., Goneska,E. and Greider,C.W. (2003) Stem–loop IV of tetrahymena telomerase RNA stimulates processivity *in trans*. *Mol. Cell. Biol.*, **23**, 5606–5613.
30. Seto,A.G., Umansky,K., Tzfati,Y., Zaug,A.J., Blackburn,E.H. and Cech,T.R. (2003) A template-proximal RNA paired element contributes to *Saccharomyces cerevisiae* telomerase activity. *RNA*, **9**, 1323–1332.
31. Chen,J.L. and Greider,C.W. (2003) Template boundary definition in mammalian telomerase. *Genes Dev.*, **17**, 2747–2752.
32. Ly,H., Xu,L., Rivera,M.A., Parslow,T.G. and Blackburn,E.H. (2003) A role for a novel 'trans-pseudoknot' RNA–RNA interaction in the functional dimerization of human telomerase. *Genes Dev.*, **17**, 1078–1083.
33. Bryan,T.M., Goodrich,K.J. and Cech,T.R. (2003) Tetrahymena telomerase is active as a monomer. *Mol. Biol. Cell*, **14**, 4794–4804.
34. Lin,J., Ly,H., Hussain,A., Abraham,M., Pearl,S., Tzfati,Y., Parslow,T.G. and Blackburn,E.H. (2004) A universal telomerase RNA core structure includes structured motifs required for binding the telomerase reverse transcriptase protein. *Proc. Natl Acad. Sci. USA*, **101**, 14713–14718.
35. Chappell,A.S. and Lundblad,V. (2004) Structural elements required for association of the Saccharomyces cerevisiae telomerase RNA with the Est2 reverse transcriptase. *Mol. Cell. Biol.*, **24**, 7720–7736.
36. Chen,J.L. and Greider,C.W. (2004) Telomerase RNA structure and function: implications for dyskeratosis congenita. *Trends Biochem. Sci.*, **29**, 183–192.
37. Dokal,I. and Vulliamy,T. (2003) Dyskeratosis congenita: its link to telomerase and aplastic anaemia. *Blood Rev.*, **17**, 217–225.
38. Mitchell,J., Wood,E. and Collins,K. (1999) A telomerase component is defective in the human disease dyskeratosis congenita. *Nature*, **402**, 551–555.
39. Vulliamy,T., Marrone,A., Goldman,F., Dearlove,A., Bessler,M., Mason,P.J. and Dokal,I. (2001) The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. *Nature*, **413**, 432–435.
40. Autexier,C., Pruzan,R., Funk,W.D. and Greider,C.W. (1996) Reconstitution of human telomerase activity and identification of a minimal functional region of the human telomerase RNA. *EMBO J.*, **15**, 5928–5935.
41. Bachand,F. and Autexier,C. (1999) Functional reconstitution of human telomerase expressed in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **274**, 38027–38031.
42. Ho,S.N., Hunt,H.D., Horton,R.M., Pullen,J.K. and Pease,L.R. (1989) Site directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene*, **77**, 51–59.
43. Feng,J., Funk,W.D., Wang,S.-S., Weinrich,S.L., Avilion,A.A., Chiu,C.-P., Adams,R.R., Chang,E., Allsopp,R.C., Yu,J. *et al.* (1995) The

human telomerase RNA component. *Science*, **269**, 1236–1241.
44. Greider,C.W. and Blackburn,E.H. (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature*, **337**, 331–337.
45. Guiducci,C., Cerone,M.A. and Bacchetti,S. (2001) Expression of mutant telomerase in immortal telomerase-negative human cells results in cell cycle deregulation, nuclear and chromosomal abnormalities and rapid loss of viability. *Oncogene*, **20**, 714–725.
46. Cerone,M.A., Londono-Vallejo,J.A. and Bacchetti,S. (2001) Telomere maintenance by telomerase and by recombination can coexist in human cells. *Hum. Mol. Genet.*, **10**, 1945–1952.
47. Bachand,F. and Autexier,C. (2001) Functional regions of human telomerase reverse transcriptase and human telomerase RNA required for telomerase activity and RNA-protein interactions. *Mol. Cell. Biol.*, **21**, 1888–1897.
48. Tesmer,V.M., Ford,L.P., Holt,S.E., Frank,B.C., Yi,X., Aisner,D.L., Ouellette,M., Shay,J.W. and Wright,W.E. (1999) Two inactive fragments of the integral rna cooperate to assemble active telomerase with the human protein catalytic subunit (hTERT) *in vitro*. *Mol. Cell. Biol.*, **19**, 6207–6216.
49. Mitchell,J. and Collins,K. (2000) Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase. *Mol. Cell*, **6**, 361–371.
50. Huard,S. and Autexier,C. (2004) Human telomerase catalyzes nucleolytic primer cleavage. *Nucleic Acids Res.*, **32**, 2171–2180.
51. Chen,J.L., Opperman,K.K. and Greider,C.W. (2002) A critical stem–loop structure in the CR4–CR5 domain of mammalian telomerase RNA. *Nucleic Acids Res.*, **30**, 592–597.
52. Ren,X., Gavory,G., Li,H., Ying,L., Klenerman,D. and Balasubramanian,S. (2003) Identification of a new RNA–RNA interaction site for human telomerase RNA (hTR): structural implications for hTR accumulation and a dyskeratosis congenita point mutation. *Nucleic Acids Res.*, **31**, 6509–6515.
53. Murnane,J.P., Sabatier,L., Marder,B.A. and Morgan,W.F. (1994) Telomere dynamics in an immortal human cell line. *EMBO J.*, **13**, 4953–4962.
54. Perrem,K., Colgin,L.M., Neumann,A.A., Yeager,T.R. and Reddel,R.R. (2001) Coexistence of alternative lengthening of telomeres and telomerase in hTERT-transfected GM847 cells. *Mol. Cell. Biol.*, **21**, 3862–3875.
55. Grobelny,J.V., Kulp-McEliece,M. and Broccoli,D. (2001) Effects of reconstitution of telomerase activity on telomere maintenance by the alternative lengthening of telomeres (ALT) pathway. *Hum. Mol. Genet.*, **10**, 1953–1961.
56. Ueda,C.T. and Roberts,R.W. (2004) Analysis of a long-range interaction between conserved domains of human telomerase RNA. *RNA*, **10**, 139–147.
57. Comolli,L.R., Smirnov,I., Xu,L., Blackburn,E.H. and James,T.L. (2002) A molecular switch underlies a human telomerase disease. *Proc. Natl Acad. Sci. USA*, **99**, 16998–17003.
58. Theimer,C.A., Finger,L.D., Trantirek,L. and Feigon,J. (2003) Mutations linked to dyskeratosis congenita cause changes in the structural equilibrium in telomerase RNA. *Proc. Natl Acad. Sci. USA*, **100**, 449–454.
59. Theimer,C.A., Blois,C.A. and Feigon,J. (2005) Structure of the human telomerase RNA pseudoknot reveals conserved tertiary interactions essential for function. *Mol. Cell*, **17**, 671–682.
60. Chen,J.L. and Greider,C.W. (2005) Functional analysis of the pseudoknot structure in human telomerase RNA. *Proc. Natl Acad. Sci. USA*, **102**, 8080–8085discussion 8077–8089.
61. Sperger,J.M. and Cech,T.R. (2001) A stem–loop of Tetrahymena telomerase RNA distant from the template potentiates RNA folding and telomerase activity. *Biochemistry*, **40**, 7005–7016.
62. Cerone,M.A., Ward,R.J., Londono-Vallejo,J.A. and Autexier,C. (2005) Telomerase RNA mutated in autosomal dyskeratosis congenita reconstitutes a weakly active telomerase enzyme defective in telomere elongation. *Cell Cycle*, **4**, 585–589.