A Self-Regulating Template in Human Telomerase

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Telomerase is a specialized reverse transcriptase (RT) containing an intrinsic telomerase RNA (TR). It synthesizes telomeric DNA repeats, (GGTTAG)n in humans, by reiteratively copying a precisely defined, short template sequence from the integral TR component. The specific mechanism of how the telomerase active site utilizes this short template region accurately and efficiently during processive DNA repeat synthesis has remained elusive. Here we report that the human TR template, in addition to specifying the repeat sequence, is embedded with a single-nucleotide signal to pause DNA synthesis. After the addition of a dT residue to the DNA primer, specified by the signaling residue 49 rA in the template, telomerase extends the DNA strand with three additional nucleotides and then pauses DNA synthesis. This sequence-defined pause site coincides precisely with the structurally defined template boundary, and cooperatively precludes incorporation of non-telomeric nucleotides from residues outside the template region. Additionally, this sequence-defined pausing mechanism prevents premature arrest of nucleotide synthesis and is the predominate mechanism for generating the characteristic 6-nt ladder banding-pattern of telomeric DNA products in vitro. In the absence of the pausing signal, telomerase stalls nucleotide addition at multiple sites along the template, generating DNA products with diverse repeat registers at the termini. Our findings demonstrate a unique self-regulating mechanism of the human TR template for high fidelity synthesis of DNA repeats.

reverse transcriptase | telomeres | ribonucleoprotein

Introduction

The ends of human chromosomes are composed of precise repetitions of a 6-nucleotide (nt) sequence synthesized by the specialized reverse transcriptase (RT), telomerase (1). The telomerase core enzyme is minimally composed of the catalytic telomerase reverse transcriptase (TERT) and the integral telomerase RNA (TR) components (2). Human TR (hTR) is a 451-nt non-coding RNA containing an exceedingly short 11-nt template which encodes specifically for the telomeric DNA repeat GGTTAG (Fig. 1A, left). The resulting highly repetitive tract of DNA is bound in a sequence-specific manner by the shelterin complex which protects natural chromosome termini from end-to-end fusions and other DNA damage responses (3, 4). High fidelity synthesis of telomeric DNA repeats by telomerase is crucial for maintaining telomere function and chromosome stability. Appending the termini of telomeres with even single-nucleotide variations in the telomeric DNA repeat sequence is sufficient for compromising the protective function of the shelterin complex, culminating in deleterious genome instability and cell death (5-8).

While TR sequences are highly divergent across taxa, the TR template itself is highly conserved (9, 10). Within vertebrates, the template sequence is conserved with the 5' boundary defined by a long-range base paired region known as helix P1, which constrains and restricts the region that functions as the template for DNA synthesis (Fig. 1A). This P1-defined template boundary relies on the physical base pairing of helix P1 as well as the length of the flanking linker to the template, functioning as a physical template boundary element (11). Extensive evidence has demonstrated the importance of the specific TR template sequence for telomerase enzymatic function, whereby alterations in the template sequence alone changes the rate and processivity of telomeric DNA repeat synthesis (12-15). Additionally, telomerase exhibited differential activity toward telomeric DNA primers with permuted sequences (16).

During telomeric DNA repeat synthesis, telomerase catalyzes nucleotide addition to the DNA primer, which forms a duplex with the RNA template within the active site. Each nucleotide addition creates a discrete RNA/DNA duplex sequence inside the binding pocket of the catalytic TERT subunit. It has remained elusive how the telomerase active site handles this growing and dynamically changing duplex during processive nucleotide addition. In this study, we investigated the active site of human telomerase utilizing its specific RNA template during nucleotide polymerization. By employing a template-free telomerase system and specific assay conditions, we discovered that the hTR template is embedded with a single-residue signal to pause nucleotide addition at an exact position, safeguarding the 5' boundary of the template region. This sequence-defined pause signal represents a unique self-regulating mechanism of the human telomerase template for the precise synthesis of the GGTTAG repeats.

Results

The duplex sequence specifies nucleotide addition pausing. To investigate how the telomerase active site interacts with different duplexes, we employed a human telomerase lacking the template region from hTR (13) and examined telomerase activity with pre-annealed RNA/DNA duplexes as substrates (Fig. 1A, right). This template-free (TF) telomerase was assayed with six permuted RNA/DNA duplexes, D1-D6, that represent the six distinct sequence registers formed during nucleotide addition for the synthesis of a GGTTAG repeat (Fig. 1B, left). Interestingly, the TF telomerase exhibited distinct extension patterns and diverse activities with each permuted duplex. In the presence of α-^{32}P-dGTP, the DNA primers from all duplex substrates were

Significance

The telomerase enzyme is essential for maintaining the replicative capacity of highly prolific cells, such as stem cells and cancer, by synthesizing telomeric DNA onto chromosome ends. Telomerase functions as an RNA-protein complex with an integral telomerase RNA component (TR). While the templates from all other reverse transcriptases (RTs) merely specify the sequence for nucleotide addition, we found that the human TR template is embedded with a single-residue pausing signal for regulating DNA synthesis. Mutation of this pausing signal alters the fundamental function of telomerase for synthesizing exact telomeric DNA repeats. This is the first instance of a single-residue pausing signal found in the RNA template of an RT.

Reserved for Publication Footnotes

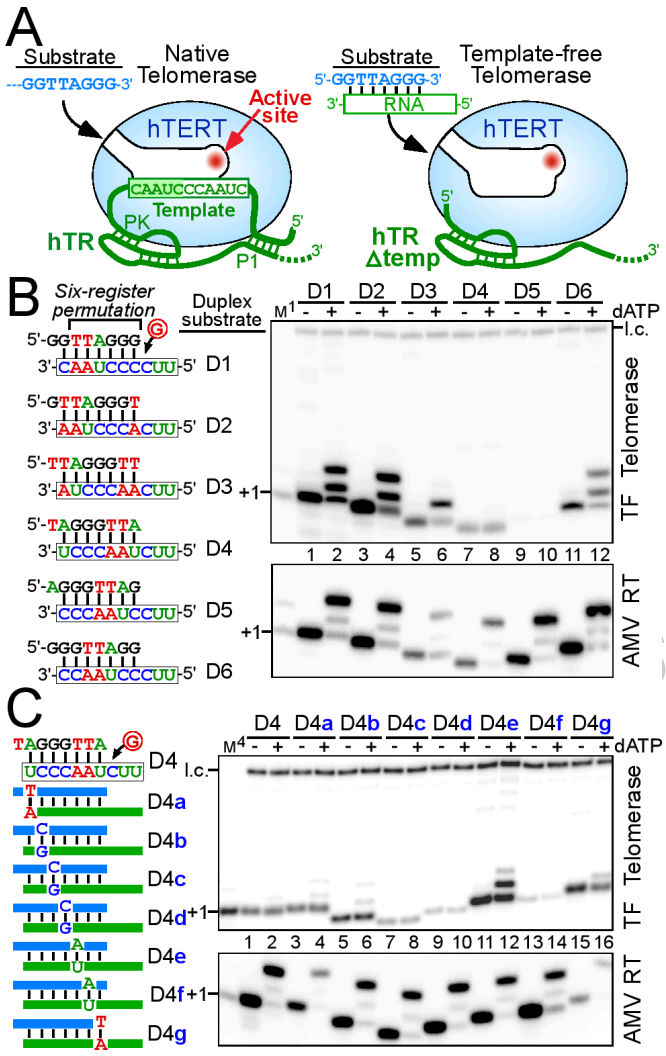

Fig. 1. . A single nucleotide in the RNA/DNA duplex signals a pause in nucleotide addition with template-free (TF) telomerase. (**A**) Schematic comparison of native (*left*) and TF (Δtemp, *right*) human telomerases. In the native telomerase, the RNA template is tethered to the 5' P1 helix and the 3' pseudoknot (PK) structures. TF telomerase was reconstituted *in vitro* with hTERT and a 5' truncated hTR (△temp) that lacks the template and the P1 helix. Substrates for the activity assay are single-stranded DNA for native telomerase or a pre-annealed RNA/DNA duplex for TF telomerase. (**B-C**, *left*) Sequences of permuted telomeric duplexes D1-D6 or D4 substitution variants. (**B-C**, *right*) Activity assay of *in vitro* reconstituted TF telomerase (*upper panel*) and AMV RT (*lower panel*) with various duplex substrates. Substrates were extended by the enzyme with α-^{32}P-dGTP in the presence (+) or absence (-) of 0.5 mM dATP as denoted above the gel. A ^{32}P end-labeled 18-mer oligonucleotide was included as a loading control (l.c.). The DNA primers GGTTAGGG (M^1) or TAGGGTTA (M^4) extended by one ^{32}P-dGTP with terminal deoxynucleotidyl transferase (TdT) were included as size markers.

extended by only one nucleotide (Fig. 1B, lanes 1, 3, 5, 7 and 11), aside from D5 which was nearly inactive (Fig. 1B, lane 9). With the addition of dATP to the reaction, D1, D2, and D6 were extended by three nucleotides, reaching the end of the 3-nt RNA template as expected of a conventional RT (Fig. 1B, lanes 2, 4 and 12). Unexpectedly, TF telomerase only extended D3 by two nucleotides and D4 by a single nucleotide (Fig. 1B, lanes 6 and 8). The differences in the extension pattern between these permuted duplexes suggested that the duplex sequence alone determined the pausing site during nucleotide addition. A recent study with TF telomerase reported a similar result (17). Furthermore, this sequence-defined pausing appears to be a telomerase-specific attribute, as AMV RT extended the DNA primers to the end of each RNA template regardless of the duplex sequence (Fig. 1B, AMV RT).

A single base pair in the duplex defines the nucleotide addition pause site. We further examined which base pair(s) in the duplex signals for this unanticipated nucleotide addition pausing. Transversion substitutions were introduced for each base pair in the D4 duplex and each variant was assayed with TF telomerase (Fig. 1C, left). Each of the D4 substitutions retained the pause signal with the exception of D4e, which had the first rA:dT base pair converted to rU:dA. D4e permitted the addition of a second nucleotide, shifting the pause site from +1 to +2 (Fig. 1C, lane 12). However, TF telomerase failed to extend the D4e duplex with a third nucleotide, pausing nucleotide addition prior to reaching the end of the RNA template. We suspected that the second rA:dT base pair, with the first rA:dT base pair mutated, gained the function as a pause signal. Indeed, transversion substitutions at both rA:dT base pairs completely abolished the sequence-defined pausing (Fig. S1A), while substitutions at the second rA:dT base pair alone failed to significantly alter the pause signal of the first rA:dT base pair (Fig. S1B). Thus, the first rA:dT base pair formed in the duplex induced a pause in DNA synthesis following the incorporation of three additional base pairs.

To discern the functional group(s) in the rA:dT base pair necessary and sufficient for inducing the sequence-defined pause in nucleotide addition, we designed variants of D4 with specific functional groups modified in the first rA:dT base pair (Fig. S2A and S2B). The TF telomerase assays with these D4 variants revealed that the transition substitutions, rA:dU, rG:dC and 7-deaza-rA:dT, which altered individual functional groups in the major and minor grooves of the duplex, had no significant effect on the position of the pause (Fig. S2C, lanes 3-8). However, the transversion substitution of the first rA:dT base pair in D4 to rU:dA effectively shifted the pause site (Fig. S2C, lane 9-10). This result suggests that any ribonucleotide purine:deoxyribonucleotide pyrimidine base pair, rR:dY, is sufficient as signal to pause nucleotide addition. Thus, it appears that the telomerase active site does not recognize specific functional groups in the signaling rA:dT base pair. The rR:dY requirement for the signaling base pair suggests an indirect readout of the pause signal by the TERT protein.

A DNA overhang alleviates nucleotide addition pausing. In the native telomerase, interactions between the DNA primer, TR template and TERT anchor sites collectively contribute to nucleotide addition processivity (13, 18). Thus, we assayed the D4 and D6 duplexes appended with either DNA or RNA overhangs to better imitate the intermediate products of a native telomerase reaction (Fig. 2A, left). While D4 harbors an effective pausing signal, D6 represents a duplex following template translocation with nucleotide addition reinitiated. Our results demonstrate that a DNA 5' overhang, and not an RNA 3' overhang, permitted partial bypass of the sequence-defined pause site for the D4 duplex (Fig. 2A, lanes 2, 6 and 10). Additional D4 substrates with various DNA overhangs or duplex lengths also displayed a similar partial bypass of the pause signal with some variation in activity (Fig. S3). The D6 duplex which lacks the pause signal, appeared unaffected by the presence of either an RNA 3' or DNA 5' overhang (Fig. 2A, lanes 4, 8 and 12). The protein-DNA interactions between the TERT anchor sites and the DNA 5' overhang presumably facilitated duplex translocation and increased nucleotide addition processivity, thus alleviating the sequence-defined pause. We further explored the RNA 5' flanking sequence on the sequence-defined pause with two RNA sequences, UGUU and CCAg (Fig. S4A). These two sequences represent the RNA 5' regions flanking the duplex prior to or following template translocation. Regardless of the flanking sequence, the pause site was retained within each of these duplexes (Fig. S4B). Thus, TERT binding

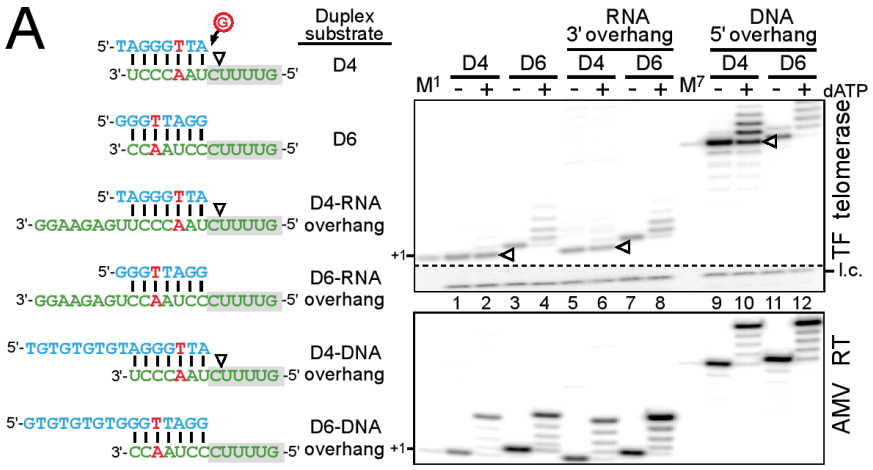

Fig. 2. . Functional assays of RNA/DNA duplex overhangs for sequence-defined pausing. (**A**) Activity assay of duplex substrates with various overhangs. Sequences of duplexes D4 and D6 appended either RNA 3' or DNA 5' overhangs (*left panel*). The sequence-defined pausing site is denoted (*white* triangles). Activity assay of duplex substrates with various overhangs (*right panel*). *In vitro* reconstituted TF telomerase or AMV RT were assayed with RNA/DNA duplexes and α-^{32}P-dGTP in the presence (+) or absence (-) of 0.5 mM dATP as denoted above the gel. A ^{32}P end-labeled 7-mer oligonucleotide is included as a loading control (l.c.). The DNA primers TAGGGTTA (M^1) and $(TG)_4$TAGGGTTA (M^7) extended by one α-^{32}P-dGTP with TdT were included as size markers. (**B**) TF telomerase is inactive with the D5 duplex that contains a DNA 5' overhang. Activity assay of *in vitro* reconstituted TF telomerase and AMV RT with duplex substrate variants (D1-D6) appended with DNA 5' overhangs. Substrates were extended by the enzyme with α-^{32}P-dGTP. A ^{32}P end-labeled 7-mer oligonucleotide was included as a loading control (l.c.).

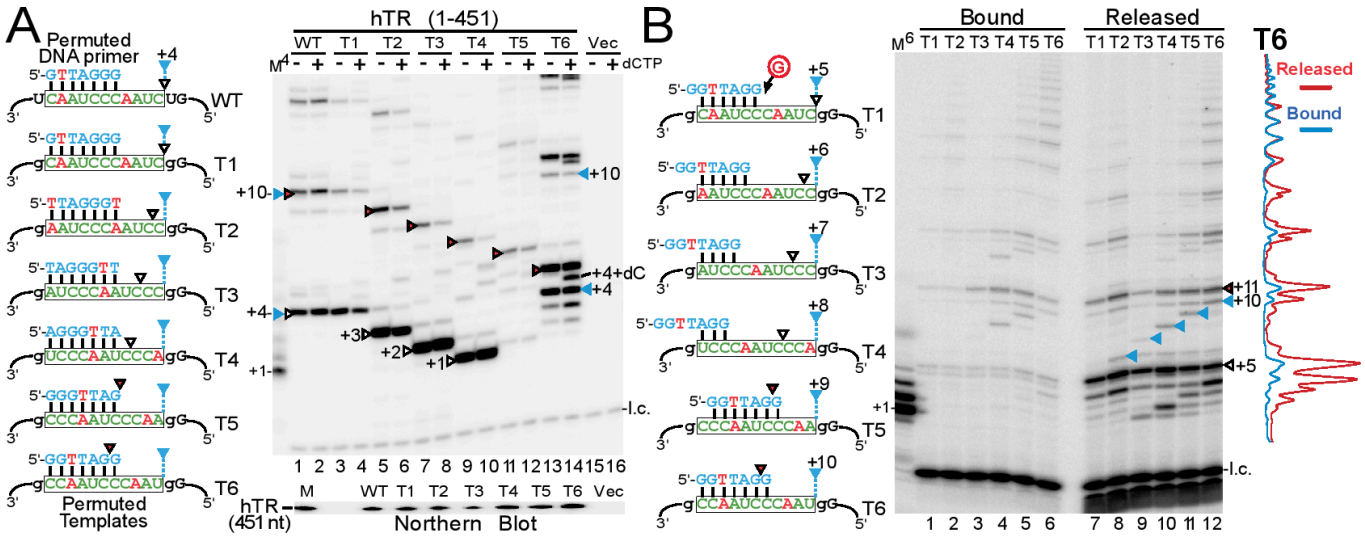

Fig. 3. *In vivo* reconstituted telomerases with permutated templates exhibit sequence-defined nucleotide addition pausing. (**A-B**,*left*) Sequences of the DNA primer and the hTR template variants for telomerase direct assay. The sequence-defined pausing sites before (*white* triangles) or after (*red* triangles) template translocation are denoted. The pausing sites at the P1-defined physical template boundary are also denoted (*blue* triangles). (**A**, *right*) Activity assay for telomerase with template permutations. Telomerases reconstituted in 293FT cells with full-length hTR containing permuted template sequences were assayed with similarly permuted DNA primers in the presence (+) or absence (-) of 0.5 mM dCTP. The DNA primer AGGGTTA extended by one α-^{32}P-dGTP with TdT was included as a size marker (M^4). The vector-only transfected cells (Vec, lanes 15-16) were included as a negative control. Northern blot for hTR co-immunopurified with FLAG-tagged TERT is shown under the activity gel. T7-transcribed hTR (451 nt) was included as a size marker (M). (**B**, *right*) Analysis of bound and released DNA products. *In vivo* reconstituted telomerases (T1-T6) were immobilized on anti-FLAG beads and assayed with a 7-mer DNA primer. DNA products released were separated from those bound to the immobilized enzyme and analyzed by gel electrophoresis. A 6-mer ^{32}P end-labeled oligonucleotide was added to the reaction mix prior to separation from the beads and a secondary 7-mer ^{32}P end-labeled oligonucleotide was added during phenol/chloroform extraction. Intensity traces of released (*red*) and bound (*blue*) products from the T6 template mutant are shown to the right of the gel. The DNA primer GGTTAGG extended by one α-^{32}P-dGTP with TdT was included as a size marker (M^6).

the DNA 5' overhang, and not the single-stranded RNA template region, appears to promote nucleotide addition processivity and potentially regulates the sequence-defined pause.

In addition to the distinct extension patterns, the six permuted RNA/DNA duplexes also exhibited markedly different activities with TF telomerase (Fig. 1B). The inactivity of D5 with TF telomerase is particularly striking and possibly results from either low binding affinity to the telomerase active site or inefficient nucleotide addition catalysis onto this substrate (Fig. 1B, lanes 9 and 10). We thus examined whether increasing the TERT binding affinity to the duplex by extending the DNA 5' overhang would increase TF telomerase activity with the D5 substrate. However, in the presence of a DNA 5' overhang, D5 remained virtually inactive (Fig. 2B, lane 5). A competitive inhibition assay suggested that the six duplexes, D1-D6, with identical DNA 5' overhangs had similar apparent binding affinities to the TERT active site

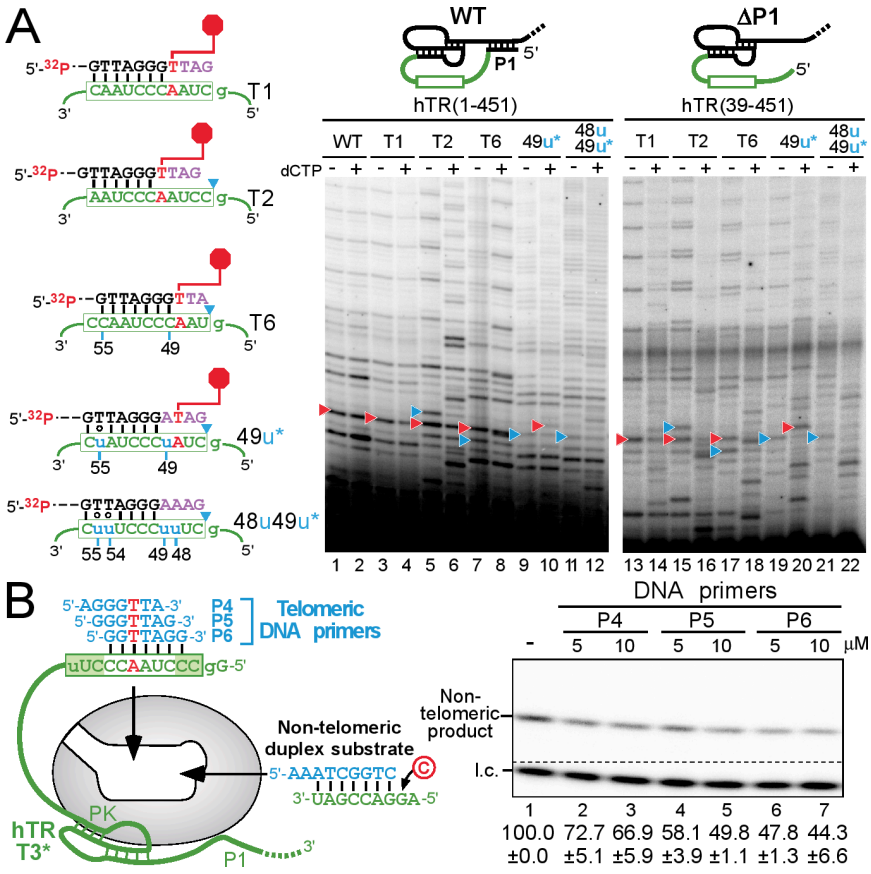

Fig. 4. Functional assays of hTR template variants for sequence-defined pausing and binding affinity of the DNA/RNA duplex to the active site. (**A**, *left*) Sequences of the DNA primer and the hTR template variants T1, T2, T6, 49u and 48u49u. The sequence-defined pausing site (*red* hexagon) and the P1-defined boundary (*blue* triangles) are denoted. The asterisk (*) denotes that the 49u and 48u49u mutants contain additional mutations (55u and 54u55u, respectively) to permit processive repeat addition. (**A**, *right*) Telomerase reconstituted *in vivo* with hTR templates variants, T1, T2, T6, 49u and 48u49u, were assayed with the 18-mer ^{32}P end-labeled DNA primer $(TTAGGG)_3$ in the presence (+) or absence (-) of 0.5 mM dCTP. The sequence-defined (*red* triangles) and P1-defined (*blue* triangles) pause sites are denoted. The secondary structure of full-length hTR (1-451) with helix P1 and truncated hTR (39-451) without helix P1 are shown above the gel. (**B**) Competitive inhibition assay of DNA primer competitors against a non-telomeric duplex substrate. Telomerase was reconstituted *in vivo* with the hTR template permuted variant T3* that lacks helix P1, the linker poly(U) tract and contains a 56u mutation. The T3* telomerase was pre-annealed with excess P4, P5, or P6 telomeric DNA primers at 5 and 10 μM as competitors when assayed against 10 μM non-telomeric substrate with α-^{32}P-dCTP. A ^{32}P end-labeled 7-mer oligonucleotide was included as a loading control (l.c.). Quantitation of the relative activity in the presence of competitor is displayed below the gel with the standard error (±) derived from two independent experiments.

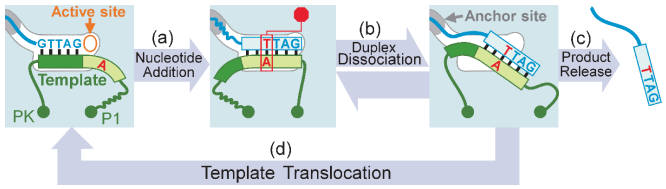

Fig. 5. A model of the telomerase sequence-defined catalytic cycle. A duplex formed from the RNA template and DNA primer is bound to the telomerase active site. *Nucleotide addition (step a)* proceeds, specified by the template sequence. After addition of three nucleotides past the pause signal (*red*), nucleotide addition is arrested. *Duplex disassociation (step b)* leads to an unbound duplex with DNA 5' overhang bound to the TERT anchor site. The strand-separation of RNA/DNA duplex results in either complete DNA *product release (step c)* or *template translocation (step d)* that aligns and regenerates the duplex bound by the active site which is ready for further *nucleotide addition (return to step a)*.

(Fig. S5). Thus, it seems that D5 inactivity is likely the result of inefficient catalysis, rather than low binding affinity to the active site.

Native telomerase exhibits sequence-defined pausing. Within the full-length native hTR, the sequence-defined pause site coincides precisely with the helix P1-defined template boundary (Fig. 1A left). To uncouple this putative sequence-defined pause from the P1-defined template boundary in the native telomerase, we reconstituted telomerase in 293FT cells with full-length hTR that harbored permuted template sequences, T1-T6. Telomerases containing hTR T1-T6 were immuno-purified from 293FT cells and assayed for activity with correspondingly permuted 7-mer DNA primers, which anneal to the same position on the template relative to the P1-defined boundary (Fig. 3A, left). The T1-T6 templates were each flanked at 5' and 3' ends by guanosine residues to further define each template region and prevent incorporation of non-telomeric nucleotides from outside the template with the omission of dCTP from the reaction. In accordance with the TF telomerase results, each template-permuted telomerases demonstrated major sequence-defined pausing at a position three nucleotides following the first rA:dT base pair (Fig. 3A, white and red triangles). In T2-T6, minor bands corresponding to pausing at the physical boundary were visible (Fig. 3 and Fig. S6, blue triangles). In the presence of dCTP, minor bands corresponding to products derived from the incorporation of non-telomeric sequences from outside the template were visible (Fig. 3A, compare even and odd lanes). This result indicated that the P1-defined boundary, when uncoupled from the sequence-defined pause, is insufficient in preventing template boundary bypass. The inadequacy of the P1-defined boundary and the predominate pause at the sequence-defined pause site were consistently observed, regardless of the length or permutation of the DNA primer assayed with each TR template permutation (Fig. S6). Thus, sequence-defined pausing effectively regulates nucleotide addition in the native telomerase.

It has been presumed that telomerase generates the characteristic 6-nt ladder banding pattern of DNA products by releasing these products at the P1-defined physical boundary during processive DNA repeat synthesis. In light of the sequence-defined pause position coinciding precisely with the P1-defined physical boundary, we investigated which mechanism is principally responsible for DNA product release. Telomerases reconstituted *in vivo* with hTR template permuted variants, T1-T6, and FLAG-tagged hTERT were immobilized on beads and assayed with a 7-mer DNA primer, 5'-GGTTAGG-3' (Fig. 3B, left). Following the telomerase extension reaction, DNA products that remained

bound to the immobilized telomerases were separated from the released products and analyzed. The results show that telomerase released DNA products at the sequenced-defined pause site and, to a slightly lesser level, at the P1-defined boundary (Fig. 3B, lanes 7-12). Therefore, nucleotide addition arrest induced by either mechanism was sufficient for product release. Notably, there was a higher accumulation of short DNA products in the released fraction (Fig. 3B, right, T6 trace lines), likely resulting from insufficient interactions between the short DNA product and TERT DNA anchor sites (19, 20).

Processive repeat synthesis does not require sequence-defined pausing. Precise product release is an important component of processive telomeric DNA repeat addition. Since sequence-defined pausing precedes product release, we explored whether this contributes to the repeat addition processivity, a unique attribute of telomerase. A telomerase template mutant 48u49u, which had the pausing signal completely eliminated, was analyzed for repeat addition processivity and DNA extension patterns (Fig. 4A, left). When boundary bypass was prevented by the absence of dCTP, template variants T2 and T6 arrested nucleotide addition at the sequence-defined pause site (Fig. 4A, lanes 5 and 7). In contrast, the 48u49u mutant paused at multiple sites along the template, producing a more evenly distributed banding pattern (Fig. 4A, lanes 11 and 12) (12). This suggests that the sequence-defined pausing mechanism prevents premature stalling during nucleotide addition and promotes pausing specifically at the sequence-defined site. Thus, sequence-defined pausing is a key determinant for generating the characteristic 6-nt ladder banding pattern of telomerase products. Surprisingly, the 48u49u mutant retained repeat addition processivity, suggesting that the sequence-defined pause is not essential for telomerase processive repeat addition (Fig. 4A, lane 11).

The complete removal of the P1-defined boundary by the deletion of helix P1 did not impair telomerase processivity, when boundary bypass was prevented by the omission of dCTP from the reaction. In this system, the T2 and T6 template variants paused nucleotide addition at the expected positions (Fig. 4A, lanes 13, 15, 17, 19 and 21). However, in the presence of dCTP, which permitted template boundary bypass, the addition of non-telomeric nucleotides from the region outside the template boundary drastically impaired telomerase processivity (Fig. 4A, lanes 14, 16, 18, 20 and 22). Therefore, while important for defining the template boundary, tethering the template to helix P1 is dispensable for telomerase repeat addition processivity as long as the non-telomeric nucleotide incorporation is prevented.

Sequence-defined pausing potentially results from catalytic deficiency. We further investigated the mechanism of sequence-defined pausing within the context of *in vivo* reconstituted telomerase. Telomerase may pause nucleotide addition either by (1) triggering a rapid release of the D5 duplex or (2) preventing catalysis with this duplex substrate. To distinguish between these two possibilities, we examined whether the telomerase active site has a lower binding affinity for the D5 duplex. A competitive inhibition assay was performed to discern the extent that each DNA primer/hTR template duplex competes for the telomerase active site against a non-telomeric duplex substrate (Fig. 4B, left). Telomerase was reconstituted *in vivo* with the hTR template permuted variant T3* that lacks helix P1 and the linker poly(U) tract and contains an A56 to U mutation to prevent DNA primer misalignment. The T3* telomerase was pre-annealed with three different single-stranded DNA primers, P4, P5 and P6, and then assayed for competitive inhibition against an exogenous non-telomeric duplex substrate (Fig. 4B, left). The reaction was performed in the presence of α-^{32}P-dCTP to permit the selective extension of the non-telomeric duplex substrate. The three duplexes formed with the P4, P5 or P6 DNA primers had similar levels of non-telomeric substrate activity inhibition, indicating comparable binding affinities to the active site (Fig. 4B, lanes 2-7). As a control, excess single-stranded DNA primers did not exhibit significant inhibition with TF telomerase that lacks any RNA template for forming a DNA/RNA duplex (Fig. S7). Thus, the inactivity of the D5 duplex observed with TF telomerase and the lower activity of the P5 DNA primer with native telomerase are likely the result of inefficient catalysis, rather than poor binding of the duplex to the telomerase activity site.

Discussion

Telomerase synthesizes telomeric DNA repeats by iteratively copying the intended template sequence from the integral RNA component. The short template region from the vastly larger TR is precisely defined to avert incorporation of non-telomeric nucleotides from the template-flanking region. The sequence-defined pausing mechanism we have discovered in human telomerase provides new insights into the template boundary definition for vertebrate telomerase. Our results show that the sequence-defined pause signal functions synergistically with the P1-defined physical boundary during the telomerase catalytic cycle. Following the processive addition of six nucleotides to the DNA primer, the first rA:dT base pair formed in the duplex signals a pause in DNA synthesis, which coincides with the P1-defined boundary (Fig. 5, step a). This pausing, while not actively promoting, permits duplex dissociation from the active site (Fig. 5, step b), which is followed by complete product release (Fig. 5, step c) or template translocation (Fig. 5, step d). Moreover, this sequence-defined mechanism stimulates high nucleotide addition processivity prior to reaching the pause site, with the loss of this mechanism resulting in a myriad of weaker pause sites throughout the template and products with heterogeneous terminal registers. Thus, this sequence-defined pausing is a key, yet previously overlooked, determinate for generating 6-nt DNA repeats with identical GGTTAG terminal sequences.

We propose that the incorporation of the first nucleotide following template translocation onto the D5 duplex is the *rate limiting step* for processive telomeric DNA repeat synthesis. In a processive human telomerase reaction, RNA/DNA duplexes formed immediately prior to and following template translocation have the sequence register GGTTAG, which is identical to the D5 duplex. In the context of TF telomerase, the D5 duplex is inactive despite the presence of a DNA 5' overhang (Fig. 2B, lane 5). Additionally, the telomerase active site itself is impartial to the duplex formed prior to and post template translocation, evidenced by the sequence of the single-stranded RNA 5' flanking the D5 duplex failing to alter sequence-defined pausing with TF telomerase (Fig. S4). However, the processive addition of DNA repeats by native telomerase requires extension of the D5 duplex formed post template translocation. Thus, inefficient nucleotide addition onto the D5 duplex post template translocation provides an explanation for the low repeat addition rate and processivity of human telomerase core enzyme.

We cannot exclude the possibility that template-containing telomerase employs additional mechanisms to facilitate extending the unfavorable D5 duplex post template translocation. Telomerase reconstituted with full-length template-permuted hTR T5 retained a low level of activity with the DNA primer GGGTTAG which forms a D5-like duplex with the hTR template, while TF telomerase is inactive with the D5 duplex (Fig. 3A, lanes 11-12 and Fig. S6B). The principle difference between TF and template-containing telomerases is the tethering of the RNA template to the core enzyme. This tethering potentially promotes nucleotide addition to the D5 duplex post template translocation (Fig. 1A, left). It has been previously proposed for *Tetrahymena* telomerase that changes in the tension of the template-flanking RNA regions during nucleotide addition facilitates proper template translocation (18). This would seemingly explain the differ-

ences between the TF and the template-containing telomerases for extending the D5 duplex. However, a human telomerase lacking the P1 helix, removing the potential tension on the 5' end of the template, retains processive repeat addition as long as template boundary bypass is precluded (Fig. 4A, lane 13). The 3' template-flanking RNA linker constrained by the pseudoknot may facilitate nucleotide addition to the D5 duplex. Future studies are necessary to explore the precise mechanism by which native telomerase extends the D5 duplex post template translocation to permit processive telomeric DNA repeat addition.

Our comparative study of purple sea urchin, *Neurospora* and *Tetrahymena* telomerases showed that sequence-defined pausing is unique to vertebrates and purple sea urchin, and not found in either ciliate or fungal telomerases (Fig. S8)(21). Interestingly, purple sea urchin telomerase appears to have a weaker sequence-defined pause than that found in human, suggesting the sequence-defined pausing has recently evolved along the vertebrate lineage. The lack of the sequence-defined pausing mechanism in fungal and ciliate telomerases is presumably offset by their distinct and more robust physical template boundaries. Ciliate and fungal TR templates have boundaries rigidly defined by protein binding and immediately adjacent helical structures, respectively (22-26). In contrast, vertebrate and purple sea urchin TR templates are tethered by a flexible linker to the distal P1 helix (11, 27). This P1-defined physical boundary is relatively less stringent and thus necessitates an ancillary mechanism, such as sequence-defined pausing, to facilitate precise synthesis of telomeric DNA repeats. Furthermore, the sequence-defined pausing mechanism correlates well with the high degree of TR template sequence conservation in vertebrate species (9, 24, 28).

The sequence-defined pause contributes to the synthesis of the exact GGTTAG register at the chromosome termini, evident by the characteristic 6-nt ladder banding pattern of telomerase-generated DNA products *in vitro*. Consistent with this finding, a previous study reported that telomerase-positive cells exhibit a markedly higher frequency of the GGTTAG register at chromosome termini (29), implying that telomerase is responsible for generating the terminal GGTTAG sequence *in vivo* rather than the result of nuclease resectioning. Interestingly, the telomeric DNA motif bound and protected by the single-stranded telomeric DNA binding protein, POT1, ends with the same GGTTAG register (30). The conservation of the terminal GGTTAG register as the product of telomerase DNA synthesis and as the moiety bound by POT1 implies a biologically functional connection and potential co-evolution between telomerase and POT1. Telomerase would synthesizes DNA with a specific terminal repeat for precise binding and protection by POT1.

The fidelity of telomeric DNA repeat synthesis by telomerase is crucial for telomere function and genome stability in germ line and stem cells as well as in cancer. Template mutations will generate mutant telomeric DNA products that will negatively affect binding by the shelterin complex (8), and impair the sequence-defined pausing and synthesis of precise telomeric DNA products by telomerase (5, 12). The self-regulating attributes of the human telomerase RNA template ensures the synthesis of exact GGTTAG repeats with exquisitely high fidelity. This unprecedented property of human telomerase demonstrates a unique means for an RT to utilize its RNA template for regulating DNA repeat synthesis.

Materials and Methods

***In vitro* reconstitution of human telomerase.** The hTERT protein was expressed in rabbit reticulocyte lysate (RRL) from pNFLAG-hTERT plasmid DNA using the TnT T7 Quick coupled kit (Promega). The hTR pseudoknot (residues 32-195 or 64-184) and CR4/5 (residues 239-328) fragments were *in vitro* transcribed, gel purified and assembled with hTERT in RRL at a final concentration 1.0 μM for 30 min at 30°C.

Additional materials and methods for telomerase activity and competitive inhibition assays are described in SI Materials and Methods.

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