Investigation of Human Telomerase Holoenzyme Assembly, Activity, and Processivity Using Disease-linked Subunit Variants*

Received for publication, November 24, 2009 Published, JBC Papers in Press, December 18, 2009, DOI 10.1074/jbc.M109.088575

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After the initial discovery of human telomerase deficiency in the X-linked form of the bone marrow failure syndrome dyskeratosis congenita, mutations in genes encoding telomerase subunits have been identified in patients with a wide spectrum of disorders. Structure/function studies of disease-linked variants of human telomerase RNA (hTR) or telomerase reverse transcriptase (TERT) have exploited in vitro reconstitution of the enzyme catalytic core and/or a PCR-amplified activity assay readout that would not reflect alterations of cellular RNP assembly efficiency, telomeric primer recognition, and/or repeat addition processivity. Here we used telomerase reconstitution in vivo and direct telomeric-repeat primer extension activity assays to compare the ribonucleoprotein (RNP) assembly and activity properties of disease-linked subunit variants in holoenzyme context. Analysis of a large panel of hTR variants revealed numerous biochemical mechanisms for telomerase loss of function, including reduced association of hTR with TERT, reduced RNP catalytic activity, or loss in fidelity of telomeric repeat synthesis. An absolute correlation exists between hTR loss of function and hematopoietic deficiency, but there is no readily apparent telomerase deficiency imposed by an hTR variant linked to pulmonary fibrosis. Some disease-linked TERT variants have altered properties of holoenzyme assembly or repeat addition processivity, but other TERT variants linked to either pulmonary fibrosis or hematopoietic deficiency retained normal hTR interaction and RNP catalytic activity. Combined with additional hTR structure/function studies, our results establish a new resolution of insight into hTR structural requirements for hTR-TERT interaction and for the catalytic cycle of human telomerase holoenzyme.

Telomeres protect chromosome ends from the fusion and degradation events that would otherwise compromise genome stability (1). In vertebrates, the dynamic chromatin required for telomere function assembles on tandem copies of the telomeric repeat TTAGGG, running 5’ to 3’ on the DNA strand with the chromosome-terminal 3’-OH (2). To compensate for under-replication of these telomeric repeats with each round of cell division, new repeats can be added by the ribonucleoprotein (RNP)2 reverse transcriptase telomerase (3, 4). Telomerase accomplishes de novo telomeric repeat synthesis using an active site constituted by telomerase reverse transcriptase (TERT) and telomerase RNA (TER). Association of human TERT (hTERT) and human TERT in vitro requires chaperone activities (5), as first shown for reconstitution of the minimal RNP by subunit expression in rabbit reticulocyte lysate (RRL). In vivo assembly of a stable RNP containing hTR and regulated association of this RNP with TERT to form the biologically active telomerase holoenzyme involve cascades of hTR processing and modification, RNP maturation, nuclear trafficking, and other events that are only partially understood (6).

Motifs within the mature 451-nucleotide hTR have been defined as required for in vivo accumulation of biologically stable RNP or for association of this catalytically inactive RNP with TERT. Accumulation of hTR depends on a hairpin-hinge-hairpin-ACA (H/ACA) motif in the 3’ half of hTR (Fig. 1A), a motif shared by hTR and a family of RNAs that guide pseudouridine formation by small nucleolar and small Cajal body RNP s (7, 8). The H/ACA motif recruits the interacting protein complex of dyskerin, NHP2, NOP10, and GAR1, all of which are component proteins of purified telomerase holoenzyme (9). All known vertebrate TER s possess an H/ACA motif (10, 11), but ciliate and yeast TER s use other motifs and associated proteins to confer RNP stability in vivo (12). Beyond the H/ACA motif, interaction of a human telomerase RNP with TERT involves two other hTR regions (Fig. 1A): that is, the 5’ half of the RNA (containing the template and pseudoknot) and vertebrate TER conserved region (CR) 4/5 (an extension of the H/ACA motif 5’ stem). These two regions of hTR independently have specific interaction with TERT in vivo and, if combined with TERT in heterologous extract such as RRL, reconstitute a minimal catalytic core of human telomerase (13).

The connection between telomerase deficiency and human disease was established in studies of X-linked dyskeratosis congenita (DC), in which amino acid substitutions of the telomerase holoenzyme H/ACA motif-binding protein dyskerin impose a reduced accumulation of telomerase RNP (7, 14). X-linked DC symptoms include a fatal loss of hematopoietic...
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renewal and, with varying penetrance, other hallmarks of proliferative deficiency such as reticulated skin pigmentation and nail dystrophy (15). X-linked DC patient fibroblasts, lymphoblasts, and lymphocytes monoaicularly express variant dyskerin and show steady-state levels of hTR that are \(\sim 20-40\%\) of that of the level of hTR accumulation in family-matched control cells (7, 16). Subsequent studies of autosomal dominant (AD) DC revealed that in several families, disease phenotypes co-segregate with heterozygous inheritance of a chromosome region including a variant allele of hTR (17). For several hTR substitution or deletion variants linked to AD DC by co-inheritance of allele and disease across an extended family, hTR loss of function is predicted to be \(\sim 50\%\), based on assays of hTR accumulation and the lack of dominant-negative impact of AD DC hTR on function of the co-expressed wild-type hTR in telomere maintenance (18, 19). A less severe loss of hTR function in AD versus X-linked DC is consistent with the later age of disease onset, although disease anticipation in successive generations of AD DC families can erase this distinction (20). For DC patients it is a combination of inherited telomere length deficiency, telomerase deficiency, and individual tissue proliferative history that together would give rise to the critical telomere shortening tightly correlated with clinical presentation of disease (21–23).

Recent studies have used a candidate sequencing approach to broaden the potential connection between human telomerase deficiency and disease (22–25). Beyond DC, mutations that change the sequence of hTR have been detected in patients diagnosed with aplastic anemia (AA) or myelodysplasia (MDS) and also in a patient diagnosed with pulmonary fibrosis (PF) without a hematopoietic deficit. This spectrum of clinical defects may be linked (23). Indeed, DC patients are at increased risk for pulmonary disease (15). However, because patients presenting with PF or AA or other hallmarks of proliferative deficiency may not be screened for the full complement of potentially telomere-linked disorders, it is difficult to gauge the extent of clinical overlap between syndromes. TERT gene mutations have been detected primarily in patients with PF, autosomal recessive DC, or AD DC (diagnosis typically based on the criteria of bone marrow failure), or mixed PF and anemia (individual members of an extended family show either pulmonary disease or anemia). Apparently less frequent mutations have been reported in genes encoding the H/ACA motif-binding proteins NHP2 and NOP10 or the telomere-binding protein TIN2 (22, 23).

Despite the appeal of a broad connection between telomerase holoenzyme subunit mutations and clinically severe proliferative deficiencies, for TERT mutations in particular, there is often not a strict linkage of the inherited mutation and disease. Many TERT gene mutations linked to PF or autosomal recessive DC may be more accurately viewed as associated with risk of disease rather than disease outright, because they do not strictly co-segregate with disease among family members (26–28). In contrast to the case for these TERT gene mutations, hTR gene mutations show apparently robust linkage of mutation and disease in the families analyzed to date. However, across the spectrum of hTR gene mutations, the relationship (if any) between genotype and phenotype is unclear. Apart from mutations that compromise accumulation of properly matured hTR \textit{in vivo} (18, 19, 29), with resulting haploinsufficiency, little is known about how disease-linked hTR variants impose a loss of function for telomerase catalytic activation. To date, most structure/function studies of disease-linked hTR or TERT variants have exploited \textit{in vitro} reconstitution of the minimal telomerase catalytic core and/or a PCR-amplified readout of telomerase activity (the TRAP assay) that are not intended to detect potential changes in cellular RNP assembly efficiency, telomeric primer recognition, and/or repeat addition processes. Telomere maintenance activity has been examined for only three hTR variants accumulated at levels comparable with wild-type hTR; a DC-linked pseudoknot disruption variant (107G→A/108C→G) and template deletion variant (Δ52–55) were both incapable of telomere elongation, whereas an AA-linked variant (72C→G) increased telomere length less than the extent accomplished by wild-type hTR (19).

Here, using a large set of patient-isolated hTR variants not compromised for accumulation \textit{in vivo}, we address whether there are mechanisms of telomerase loss of function that correlate specifically to an initial disease diagnosis of DC, AA, MDS, or PF. For comparison, we examined a sampling of TERT variants identified in association with DC, PF, or mixed PF and anemia. Because \textit{in vitro} reconstitution of hTR and TERT alone does not produce the biologically functional state of telomerase holoenzyme harboring a monomer of hTR, a monomer of TERT, and several H/ACA motif-associated proteins (9, 19, 30, 31), we used \textit{in vivo} reconstitution to assemble subunit variants into active enzyme. We used tag-based affinity purification to quantify hTR-TERT interaction and used telomeric-repeat primer extension assays to monitor the catalytic activity of purified RNPs. These unprecedented studies of disease-linked telomerase subunit variants uncover several mechanisms by which variants of hTR impose telomerase deficiency and strengthen the relationship between hTR loss of function and hematopoietic disease. Combined with additional mutagenesis of a functionally critical region of hTR region with ambiguous secondary structure, we refine an overall map of hTR and TERT structural requirements for holoenzyme assembly and catalytic activity.

**EXPERIMENTAL PROCEDURES**

\textbf{Disease Variant Synopsis—}Unless noted otherwise, all hTR and TERT variants were detected in patients in heterozygous combination with a wild-type allele. AD DC hTR variants 107G→A/108C→G and Δ96–97 were identified in index cases with severe hematopoietic disease and premature telomere shortening in families showing generational disease anticipation (17, 20). The 305G→A mutation was identified in \textit{“DC-like”} disease with childhood onset of hematopoietic deficiency and premature telomere shortening (32). The hTR variants 72C→G and Δ110–113 were identified in index cases with AA in families showing generational disease anticipation; premature telomere shortening was less severe in association with the 72C→G variant (20, 33). The hTR Δ110–113 variant was also found in association with AA, MDS, and acute myeloid leukemia in members of an independent family (34). The hTR variants Δ28–34, 116C→U, 117A→C, and 204C→G were identi-
Whole-cell extracts were made by two cycles of freeze-thaw transfection control. The hTERT 143G→A variant was identified in a patient with hypoplastic MDS and premature telomere shortening (20). The hTERT P33S and L55Q PF-associated variants were identified in patients from families with incomplete penetrance of disease due to mutation inheritance; no anemia was reported in either family (26, 27). TERT R865H was identified in a PF patient from a family with some members showing pulmonary disease and others anemia, with incomplete penetrance of disease due to mutation inheritance (27). Heterozygous occurrence of TERT P721R was identified in a 2-year-old girl with apparent autosomal recessive DC; unaffected family members including her mother were also heterozygous for the allele (40). Heterozygous occurrence of TERT F1127L was identified in an 8-year-old boy with bone marrow failure and developmental delay; his heterozygous carrier mother was symptom-free (28).

**Cell Culture and Expression Constructs**—Human 293T cells and VA13+TERT cells (VA13 cells with an integrated vector for constitutive expression of N-terminal 1× FLAG-tagged TERT) were transfected using calcium phosphate. The transiently transfected hTR constructs were based on pBSU3hTR500, which contains the RNA polymerase II human U3 small nuclear RNA promoter upstream of mature TER sequence and 500 bp of the downstream genomic region following the mature TER 3′ end (29). The transiently transfected 3xF-TERT expression construct encodes TERT fused to an N-terminal tag with three tandem copies of the FLAG epitope (19). Sequence substitutions were introduced by site-specific mutagenesis of double-stranded DNA plasmid vector and confirmed by DNA sequencing. A construct expressing Tetrahymena thermophila TER under control of the RNA polymerase III human U6 small nuclear RNA promoter was included as a transfection control.

**Extract Preparation and Holoenzyme Affinity Purification**—Whole-cell extracts were made by two cycles of freeze-thaw lysis in HLB buffer (20 mM HEPES at pH 8.0, 2 mM MgCl2, 10% glycerol, 0.2 mM EGTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) followed by the addition of 0.4 M NaCl and centrifugation to clear the extract. Affinity purifications were performed in cell extract adjusted to 150 mM NaCl. FLAG M2 monoclonal antibody resin (Sigma) was added followed by end-over-end incubation at 4 °C for 2 h. Beads were washed 3 times with HLB containing 150 mM NaCl, 0.1% Triton X-100, and 0.2% CHAPS at room temperature and then used directly for RNA extraction or activity assays. Immunoblots to detect 3xF-TERT used FLAG M2 monoclonal antibody (Sigma) and were imaged using an Odyssey system (LI-COR).

**RNA Detection**—Total RNA was prepared from cell extract by TRIzol (Invitrogen). RNA was extracted from affinity-purified telomerase preparations also using TRIzol. Northern blots were performed using equivalent cell extract aliquots normalized by total protein (input) or equivalent aliquots of purified samples (bound) resolved on a 5% (19:1), 0.6× Tris borate-EDTA, 7 M urea gel. After transfer to Hybond N+ (GE Healthcare), hTERT was detected using an oligonucleotide equally complementary to all variants and end-labeled with [γ-32P]ATP. A nonspecific cross-reacting RNA served as the loading control for equal input of total RNA. A recombinant fragment of hTERT was added to purified RNPs before RNA extraction to serve as a recovery control for equivalent loading of purified samples. The transfection control was also detected with an end-labeled complementary oligonucleotide. All Northern blots were imaged using a Typhoon system (Amersham Biosciences).

Northern blot signal intensities were quantified using ImageQuant software (GE Healthcare). Quantification of hTERT signal was performed using a box of fixed area to calculate signal intensity, which was first subtracted for background signal of the same area and then normalized to the signal intensity of loading control (for input samples) or recovery control (for co-IP samples). Values calculated from the same exposure of the same blot were then divided by signal from wild-type hTERT to give the numbers shown beneath the figure panels of hTERT input and hTERT co-IP. The hTERT co-IP adjusted for differences in hTERT input is given as normalized co-IP. Note that mature hTERT migrates as a doublet due to partial folding despite denaturing gel electrophoresis. Both migration forms of hTERT 305G→A were included in quantification; these were reproducibly observed across several transfections using a plasmid confirmed to have the intended DNA sequence.

**Activity Assays**—Primer extension activity assays were performed in 20-μl reaction volumes with purified RNP washed into HLB buffer with no NaCl. The reaction buffer contained additional final concentrations of 50 mM Tris acetate (pH 8.0), 4 mM MgCl2, 5 mM dithiothreitol, 250 μM dTTP, 250 μM dATP, 5 μM unlabeled dGTP, 0.5 μl of 3000 Ci/mmol ([α-32P]dGTP (PerkinElmer Life Sciences), and 500 nM (T2AG3)3. Reactions were incubated at 30 °C for 60 min and stopped by adding 80 μl of TE buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA). Products were extracted and precipitated with 10 μg each of tRNA, linear polyacrylamide, and glycogen as carriers. Products were then resolved on a 10% (19:1), 0.6× Tris borate-EDTA, 7 M urea gel. Dried gels were imaged by Typhoon. For activity assays investigating template boundary bypass, dCTP was included in the reaction. For reactions using radiolabeled dGTP, assays included 250 μM dCTP. For reactions with radiolabeled dCTP, unlabeled dGTP was included at a final concentration of 250 μM, and 2 μl of 3000 Ci/mmol ([α-32P]dGTP (PerkinElmer Life Sciences) was added without additional, unlabeled dGTP.
RESULTS

Characterization of Disease-linked Variants of hTR—Human telomerase deficiency can be caused by a reduced steady-state level of hTR, for example, in patients with X-linked or some cases of AD DC (14). However, numerous disease-linked hTR variants are predicted to or have been shown to accumulate to normal level, consistent with sequence substitutions or deletions outside the H/ACA motif regions necessary for hTR RNP biogenesis and stability (Fig. 1A). Whether these hTR variants impose telomerase loss of function by reduced hTR-TERT interaction, loss of active site function, and/or loss of repeat addition processivity remains unknown. To address the biochemical mechanisms underlying telomerase deficiency caused by disease-associated hTR variants, we refined methods for 1) in vivo reconstitution of variant hTR (or TERT) into telomerase holoenzyme, 2) affinity purification of telomerase holoenzyme with the subunit variant by virtue of tagged TERT, 3) quantification of hTR-TERT interaction, and 4) activity assays of purified holoenzyme with direct extension of a telomeric-repeat primer. We tested a panel of hTR variants associated in heterozygous combination with wild-type hTR with DC, AA, MDS, or PF (see disease variant synopsis under “Experimental Procedures”; these variants are indicated by dark (DC), intermediate (AA/MDS), light (PF), or light and underlined (PF or anemia) font in Fig. 1A, with a polymorphism indicated in italic font).

TERT Interaction Analysis for hTR Variants of the Template/Pseudoknot Region—Most disease-linked variants of hTR affect residues in the 5’ half of the mature RNA harboring the template and pseudoknot, preceding the 3’ H/ACA motif required for hTR accumulation (Fig. 1A). Apart from hTR variants with an altered template, which lack accumulation and/or biological activity (19), other hTR variants alter base-pairing of a stem. These variants occur throughout the template/pseudoknot-enclosing P1a and P1b (Δ28–34, 37A→G, 204C→G), template-adjacent P2a (72C→G, 143G→A), and the pseudoknot (Δ96–97, 98G→A, 107G→A/108C→G, Δ110–113, 116C→U, 117A→C). The exception hTR 58G→A has been classified as a polymorphism (see disease variant synopsis under “Experimental Procedures”). We overexpressed this set of hTR variants by transient transfection in either VA13 cells with an integrated FLAG-TERT expression vector or 293T cells with co-transfected triple FLAG-tagged TERT (3xF-TERT). VA13 cells lack endogenous hTR (41), such that all hTR necessarily derives from recombinant hTR expression. In 293T cells, transiently expressed hTR accumulates at much higher than endogenous levels such that purification of co-expressed TERT indicated by dark (DC), intermediate (AA/MDS), light (PF), or light and underlined (mixed PF or anemia) fonts with a polymorphism indicated in italic font.

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Figure 1A shows the telomerase architecture, hTR sequence variants, and interaction of hTR variants with TERT. A mature hTR folding establishes a 5’ region, closed by a paired stem and containing the template and pseudoknot, and a 3’ region, bounded by the two stems of the H/ACA motif and containing CR4/5. CR4/5 provides the primary affinity of TERT interaction, with additional an interaction(s) occurring in the template/pseudoknot region. The template/pseudoknot region sequence and pairing are shown in expanded detail, with the numbering of positions within the mature full-length hTR. Variants in the template/pseudoknot region studied here are
recovered almost exclusively recombinant hTR and recombinant RNP catalytic activity.

To determine whether hTR variants in the template/pseudoknot region were compromised for hTR-TERT interaction, we used FLAG antibody resin to purify TERT complexes. Levels of hTR in input extracts and in holoenzyme affinity purified by TERT were quantified by Northern blot hybridization using an oligonucleotide complementary to the template region of hTR (Fig. 1, A and B). A nonspecific cross-reacting RNA in the input extracts was used as the loading control for the input samples. A transfection control was also detected in input samples to verify comparable transfection efficiency, and a recovery control was added to purified RNP before extraction to control for RNA recovery. As expected, levels of hTR in the input extracts were similar (Fig. 1, A and B, lane 1) or recombinant hTR but no tagged TERT (Fig. 1C, lane 2). We quantified hTR-TERT association by determining a normalized co-IP value for each hTR variant (see “Experimental Procedures” for quantification of hTR co-IP and input-adjusted normalized co-IP values). Overall, no substantial inhibition of TERT interaction was observed for the template/pseudoknot region hTR variants. For data shown in Fig. 1, the minimal recovery of an hTR variant was 59% that of wild type (Fig. 1B, lane 10, Normalized co-IP of hTR 107G→A/108C→G in VA13+TERT). This retention of TERT interaction by template/pseudoknot region hTR variants is consistent with the predominant role of the CR4/5 region in mediating hTR assembly with TERT (13). Some differences between samples are expected due to technical variations in handling across the large number of cells and samples processed in parallel, but over numerous experimental repetitions of the hTR-TERT interaction assays performed using independent transfections of both 293T and VA13+TERT cells, none of the hTR variants had a reproducible decrease or increase in TERT interaction (data not shown). From a conservative perspective, we note that minor differences (well within a 2-fold difference) would not have been discriminated in the background of experimental variance; however, differences representing a modest change to 50% that of the wild-type signal were readily discerned (see the example of TERT L55Q below; see “Discussion”).

Catalytic Inhibition by hTR Variants of the Template/Pseudoknot Region—The purified holoenzymes assembled on hTR variants were next assayed for telomerase catalytic activity by primer extension reactions with radiolabeled dGTP, dTTP, dATP, and the telomeric-repeat DNA substrate (T₆₋₆). In contrast to hTR-TERT interaction, hTR function in telomerase catalytic activity varied greatly across the template/pseudoknot region variants (Fig. 2). Only two hTR variants assembled telomerase holoenzymes with activity comparable with wild type; that is, the 58G→A polymorphism and the 37A→G variant unique in its association with PF without anemia (see the disease variant synopsis under “Experimental Procedures”). Activity of hTR 37A→G and hTR 58G→A holoenzymes was robust both in the overall amount and in the profile of repeat addition processivity, independent of whether assembly was performed in VA13+TERT cells (Fig. 2A, lanes 2, 4, and 5) or in 293T cells (Fig. 2B, lanes 3, 5, and 6). Over numerous repetitions of the hTR 37A→G holoenzyme assembly, affinity purification, and primer extension activity assay, we found no reproducible difference from wild type (data not shown). A TRAP assay of RRL-reconstituted minimal RNP with 37A→G hTR reported catalytic activity at 70% that of the wild-type level (27), a difference that could have been within the range of experimental variability of different subunit preparations. We
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conclude that among the disease-linked template/pseudoknot hTR variants, only the 37A→G variant has little if any difference from wild-type hTR in telomerase catalytic activation.

Minimal if any catalytic activity was detectable for holoenzymes assembled with the majority of pseudoknot variants (Δ96–97, 98G→A, 107G→A/108C→G, Δ110–113). Two AA-associated pseudoknot variants, 116C→U and 117A→C, imposed only partial inhibition of repeat synthesis activity (Fig. 2, A, lanes 12 and 13, and B, lanes 13 and 14). The residual catalytic activity of holoenzymes assembled by the hTR variants 116C→U and 117A→C is well above the undetectable (or less than 1% of wild type) activity reported for these hTR variants reconstituted with TERT in VA13 cells and assayed by TRAP of cell extract (36). Disruption of the P2a stem contiguous with the pseudoknot was severely inhibitory (Δ79), whereas P2a.1 stem disruptions (72C→G, 143G→A) and P1 stem disruptions other than 37A→G (28–34, 204C→G) were intermediate in impact (Fig. 2). The relatively less severe loss of function for hTR 72C→G holoenzyme is consistent with the relatively less severe telomere shortening in AA patients with this hTR variant (20) and with its intermediate level of function in telomere maintenance (19).

Overall, all hTR variants associated with hematopoietic disease showed a loss of function for telomerase catalytic activation, albeit to varying extent. All of the variants identified in association with DC showed severe loss of function (107G→A/108C→G, Δ96–97), whereas variants identified in association with AA ranged from severe to modest in the loss of function (Δ28–34, 72C→G, Δ79, Δ110–113, 116C→U, 117A→C, 204C→G). The single hTR variant identified in a family with members reported to have PF or anemia showed a severe loss of function (98G→A), and the single variant associated exclusively with MDS showed a partial loss of function (143G→A). Only an hTR variant categorized as a polymorphism (58G→A) and the hTR variant associated with PF without anemia (37A→G) were not reproducibly distinguishable from wild type in holoenzyme catalytic activity.

Template Boundary Bypass—Of the holoenzymes assembled with hTR template/pseudoknot-region variants, only holoenzyme with the P1 stem disruption Δ28–34 showed a dramatically altered profile of product synthesis (Fig. 2, A, lane 3, and B, lane 4). Although repeat addition processivity remained robust, predominant products of the repeat addition ladder were offset by an increase of one nucleotide. Given the role of P1 in halting the active site at the template 5′ boundary (42), the change in product profile suggests an increased tendency toward template 5′ boundary bypass. Copying past the template 5′ boundary can proceed for only a single additional nucleotide in standard reactions with dGTP, dTTP, and dATP (Fig. 3A). The additional nucleotide could be added to and removed from a substrate in repeated cycles of synthesis and cleavage by same telomerase active site (43).

To investigate potential alteration of the template 5′ boundary by hTR variants in P1 (Fig. 3A), we performed parallel activity assays in the presence and absence of dCTP. If dCTP is included in the activity assay reaction and the 5′ template boundary is not enforced, synthesis can proceed through the template-adjacent single-stranded region toward P1 (Fig. 3A). In reactions without dCTP, hTR Δ28–34 holoenzyme generated a product ladder offset to start at primer +5 relative to the predominant product accumulation of wild-type enzyme beginning at primer +4 (Fig. 3, A and B, compare lanes 3 and 4 and note the arrows in lane 4 that indicate examples of offset products). A lesser extent of this same shift in product distribution was evident for the hTR variant 204C→G, which also disrupts P1 (Fig. 3B, compare the ratio of +4 to +5 products across lanes 3–7). In reactions that included dCTP, hTR Δ28–34 and 204C→G holoenzymes showed reduced overall product yield and notable depletion of products in the repeat register of primer +5 (Fig. 3B, lanes 9 and 12). This product depletion could derive from copying an extended template to generate +dCTP products of lengths up to primer +8 or +9 (Fig. 3B, arrows in lane 9 indicate these offset products).

To directly test whether holoenzymes associated with disease-linked variants of hTR that disrupt P1 have a decreased fidelity of repeat synthesis, we performed primer extension reactions using unlabeled dGTP and radiolabeled dCTP. Incorporation of dCTP is limited by the low nucleotide concentration used to minimize dilution of the radiolabel (see “Experimental Procedures”). Nonetheless, holoenzymes assembled with Δ28–34 or 204C→G hTR but not with wild-type, 37A→G, or 58G→A hTR showed detectable incorporation of dCTP, with synthesis of a predominant product migrating as primer +8 (Fig. 3B, lanes 14 and 17). This product would result from copying the entire single-stranded region 5′ of the normal template boundary as an extended template (Fig. 3A). Consistent with disrupted formation of P1 and the ensuing template boundary defect, the Δ28–34 and 204C→G hTR variants showed altered migration of a subset of the hTR detected by Northern blot (this is particularly evident in total RNA of 293T cells: see Fig. 1C, hTR input lanes 4 and 16). We speculate that these variants adopt an alternative fold of unpaired P1 sequence(s).

Investigation of hTR Secondary Structure within CR4/5—In addition to sequence variants in the 5′ template/pseudoknot region, sequence variants in the 3′ H/ACA domain have been reported in association with disease. Many of these have been shown to or are likely to impact hTR accumulation. The structural requirements for hTR folding in the CR4/5 region have not been completely defined. The CR4/5 region (Fig. 4A) binds TERT directly, and it can reconstitute activity when combined with TERT and a physically separate template/pseudoknot region fragment in RRL (13, 44). Some conserved residues of vertebrate TERs (10, 11) fall within the P6.1 stem and loop, whereas other residues of hTR conserved among most vertebrates are positioned in flanking regions of ambiguous secondary structure depicted as a large internal loop (Fig. 4A).

We first adopted a compensatory mutagenesis approach to assess whether the internal loop region contains an additional stem with a functional requirement for base pairing rather than primary sequence. Three alternative base-pairing configurations of internal loop residues could occur (Fig. 4A); stem A (formed by residues shown in triangles), stem B (formed by residues shown in ovals), and stem C (formed by residues
shown in rectangles). We introduced single nucleotide substitutions alone and in compensatory combinations that would discriminate the disruption and repair of each putative stem (Fig. 4A, substituted positions are indicated in bold font with pointer arrows). Each substituted hTR was assembled with co-expressed 3xF-TERT in 293T cells, and holoenzymes were affinity-purified using FLAG antibody resin. Several of the hTR sequence substitutions inhibited telomerase catalytic activity, in particular those that altered conserved residues G254, C255, G315, and C320 (Fig. 4B; lanes 4, 5, 10, and 11). None of the individual substitutions that reduced holoenzyme activity was restored in activity when tested with a compensatory combination (Fig. 4B, lanes 6 and 12). Although we cannot exclude the possibility of a stem functioning in a sequence-specific manner, it seems plausible that in the holoenzyme context CR4/5 does not adopt additional pairing of internal loop residues.

Functional Analysis of CR4/5 Substitutions—To quantify the extent of full-length hTR interaction with TERT that is dependent on CR4/5, we first tested TERT co-purification of hTR lacking the entire CR4/5 region (Fig. 5A; deletion of residues 225–348). This internally truncated hTR accumulated robustly (Fig. 5B, lanes 4 and 5) but was greatly reduced in association with TERT (Fig. 5B, compare TERT co-IP of wild-type hTR in lane 3 to TERT co-IP of hTR ΔCR4/5 in lane 5). As expected, the low level of purified hTR ΔCR4/5 holoenzyme demonstrated undetectable catalytic activity (Fig. 5C, lane 5).

Within CR4/5, the DC-linked hTR variant 305G→A disrupts the P6.1 stem. We examined telomerase inhibition by the 305G→A disease variant and a panel of other single-nucleotide substitutions of conserved sequence for comparison (Fig. 5A, positions of single-nucleotide substitution are indicated by bold font and arrows). We also addressed the sequence versus structural significance of human P6.1 through investigation of a more extensive stem disruption (312U→A/313C→G/314U→A; Fig. 5A), alone or in combination with the compensatory stem-repair substitution (P6.1 unpair and P6.1 repair, respectively). Holoenzymes were assembled with these substituted hTRs by reconstitution in 293T cells. In previous studies of murine TER, unpairing of the P6.1 stem eliminated the catalytic activity of minimal or holoenzyme RNP assayed by TRAP; it also reduced CR4/5 binding to TERT in RRL (44). Also, combined substitution of both conserved P6.1 loop residues (equivalent to hTR 307U→C and 309G→A)
eliminated minimal RNP activity assayed by TRAP without preventing CR4/5 binding to TERT (44).

Substitutions of individual conserved residues generally reduced but did not preclude hTR-TERT interaction (Fig. 5B, lanes 6–10) or holoenzyme catalytic activity (Fig. 5C, lanes 6–10). No individual substitution of an internal loop residue compromised TERT binding or holoenzyme catalytic activity to the same extent as the entire CR4/5 deletion. Reproducibly, the substitutions 255C→G and 301A→U immediately flanking P6a (Fig. 5A) were less inhibitory for TERT binding and holoenzyme catalytic activity than the other internal loop substitutions 254G→C, 315G→C, and 320C→G (Fig. 5B, hTR co-purification is greater in lanes 7 and 8 than in lanes 6, 9, and 10; Fig. 5C, holoenzyme activity roughly parallels the amount of hTR co-purified with TERT). These results suggest that the internal loop provides a contact surface for TERT binding. Some positions in the internal loop are likely to contact TERT directly, whereas others may indirectly promote an internal loop conformation that is accessible for TERT binding. Although it is somewhat surprising that the conserved-residue substitutions 255C→G and 301A→U have such modest impact on hTRE-hTR interaction and holoenzyme catalytic activity, we note that each of these substitutions fortuitously precludes extension of P6a base-pairing into the internal loop. Thus, the role of sequence conservation at positions immediately flanking P6a could derive from the need to configure the overall architecture of the internal loop.

In contrast to the internal loop substitutions, substitutions that unpaired P6.1 severely compromised holoenzyme catalytic activity. The disease-linked 305G→A variant greatly reduced TERT binding and catalytic activity (Fig. 5, B and C, lane 11). Some mature 305G→A hTR migrated atypically in denaturing gel electrophoresis despite the lack of any other sequence disruption in the expression plasmid (data not shown). The triple-nucleotide P6.1 unpair substitution also greatly reduced TERT binding, but combination of this substitution with the compensatory substitution to repair the P6.1 stem rescued both TERT interaction and holoenzyme catalytic activity (Fig. 5, B and C, lanes 12 and 13). Individual substitutions of two of the P6.1 loop residues had functional consequences distinct from any of the other substitutions. The two residues flanking the stem-capping non-canonical G-U wobble pair are both absolutely conserved among vertebrate TERs, whereas the central loop residue (G308 of hTR; Fig. 5A) is less conserved. The 308G→A hTR assembled a holoenzyme with an efficiency and activity indistinguishable from wild-type hTRE. Although the flanking substitutions 307U→C and 309G→A imposed a slight if any reduction in hTRE-TERT interaction, each severely inhibited catalytic activity (Fig. 5, B and C, lanes 14–16). These findings indicate that the functional significance of residues in the P6.1 loop mirrors their extent of evolutionary conservation, unlike the case for residues of the internal loop. Also, another distinction from internal loop residues is that the conserved P6.1 loop residues play a critical role in telomerase activation beyond the requirement for assembly of hTRE and TERT.

**Functional Analysis of Patient-isolated TERT Variants**—For comparison with the disease-linked hTR variants studied above, we characterized a sampling of the disease-linked variants of TERT expressed in patients in heterozygous combination with a wild-type TERT allele (see the disease variant synopsis under “Experimental Procedures”). Some disease-associated mutations of the TERT locus preclude expression of a full-length protein or encode a protein without catalytic activity.
However most, including those studied here (Fig. 6A), introduce single amino acid substitutions with unpredictable functional impact. Variant 3xF-tagged TERT proteins were overexpressed with wild-type hTR in 293T cells and purified using FLAG antibody resin for assays of holoenzyme assembly and catalytic activity. TERT-hTR interaction was detected for all variants, with a modest reduction of hTR co-IP with the TERT L55Q variant (Fig. 6B). Over numerous experimental repetitions, none of the other variants had a reproducible difference from wild type in assembly with hTR (data not shown).

In addition, all TERT variants assembled holoenzymes with catalytic activity above the background recovered in the absence of 3xF-TERT (Fig. 6C). Only TERT L55Q purification yielded substantially less catalytic activity (Fig. 6C, lane 4), paralleling the decrease in hTR co-IP.

Across numerous experimental repetitions, L55Q TERT co-IP of hTR and catalytic activity were both an average of ~50% of wild type, an only 2-fold difference in function (data not shown). Consistent with these results, TERT L55Q, reconstituted as minimal RNP in RRL and assayed by primer extension, had an estimated ~35% of wild-type TERT catalytic activity (26). Curiously, R865H TERT holoenzyme had robust catalytic activity but reduced repeat addition processivity (Fig. 6C, lane 6). This reproducible change in repeat addition processivity would have unknowingly affected the TRAP assay of minimal RNP reconstituted in RRL, which yielded a reported ~25% of wild-type catalytic activity for the TERT R865H variant (27). Other TERT variants P33S, P721R, and F1127L were not compromised in either hTR interaction or holoenzyme catalytic activity in quantifications averaged over numerous independent experimental repetitions (data not shown).

Comparing across the panel of disease-linked TERT variants, unlike the case for hTR variants, there is not a strong correlation between disease association and loss of function for telomerase catalytic activation. The TERT variant R865H is linked to PF or anemia across extended family members; this TERT assembles holoenzyme with robust activity but reduced repeat addition processivity. Of the TERT variants identified in association with PF without anemia, TERT L55Q showed ~50% of wild-type hTR binding and catalytic activation, whereas TERT P33S showed no reproducible difference from wild-type TERT. Finally, neither of the DC-associated TERT variants had a difference in function from wild-type detectable within the mar-
gin of experimental reproducibility. Overall, these modest or undetectable telomerase functional deficiencies are consistent with the incomplete penetrance of disease among family members that are heterozygous carriers of the disease-linked allele (see disease variant synopsis under “Experimental Procedures”). Interestingly, specifically for the three TERT variants found to not substantially compromise telomerase catalytic activation, there are symptom-free mutation carriers older in age than family members affected with disease (see disease variant synopsis and references under “Experimental Procedures”).

**DISCUSSION**

**Genotype to Phenotype Correlation for Disease-linked Variants of hTR and TERT**—All hTR variants linked to hematopoietic disease showed loss of function for telomerase catalytic activation. However, across the distinct biochemical mechanisms found to underlie telomerase deficiency, there is no specific correlation between the mechanism for loss of function and disease presentation. Considering hTR variants associated with DC, AA, or MDS, telomerase loss of function involved reduced hTR-TERT interaction (305G→A), altered template use (H900428–34, 204C→G), or partial to severe loss of holoenzyme catalytic activity (the remaining variants). There is some tendency toward increasing severity of disease with increasing severity of telomerase deficiency. As the exception, the PF-associated 37A→G hTR variant appeared to be wild type in TERT binding, telomerase catalytic activation, repeat addition processivity, and even template 5’ boundary definition. From a conservative perspective, very minor differences in holoenzyme catalytic activity would not have been discriminated within experimental variance. Also, there could be a somatic cell context in which the hTR 37A→G variant does impose telomerase deficiency through a mechanism not recapitulated in our studies here. However, it seems possible that the hTR 37A→G variant is either non-causal for disease or dependent on multigenerational inheritance for disease manifestation. In the clinical identification of hTR 37A→G (27), two family members had pulmonary fibrosis (a grandfather aged 83 at death and a mother aged 64 at death) and two family members had the 37A→G hTR variant (the mother and a 43-year-old son without pulmonary fibrosis); the hTR variant carrier status of other family members remains unknown.

Disease-linked mutations of the TERT locus can impose severe loss of function, evident from studies of the TERT K902N variant responsible for multigenerational anticipation of AD DC in a family with premature graying, AA, and fibrosis.

**FIGURE 6. Characterization of disease-associated variants of TERT.**

* A, selected disease-associated TERT variants are schematized by their location within TERT domains: TEN, essential N-terminal domain; TRBD, high affinity RNA binding domain; RT, active site domain; CTE, C-terminal extension. B and C, TERT-hTR interaction and holoenzyme catalytic activity were assayed in RNP purifications from aliquots of the same transfected 293T cell extracts. Lane 1 samples are a negative control of purification from extract of cells transfected with only the hTR expression plasmid. Samples in all other lanes have co-transfected wild-type hTR and the indicated TERT. Panels in the TERT-hTR interaction assay are as described in Fig. 1, with the bold number indicating a reproducible, at least 2-fold difference from wild-type. LC, loading control; RC, recovery control.
of the lung and liver (45). For the TERT variants studied here, holoenzyme functional deficiencies were surprisingly modest. Again, from a conservative perspective, minor differences would have not been discriminated within experimental variance. However, telomere lengths in patients with most TERT variants are not well characterized, particularly for disease-relevant cell types other than lymphocytes; it is possible that the extent of telomere shortening is not as severe as in patients with disease linked to mutation of hTR. Our findings suggest that the significance of some TERT sequence changes may not be evident even with direct primer extension assays of reconstituted holoenzyme. Given the largely unaccounted complexity of TERT post-translational regulation in human somatic cells (6), some TERT variants may have altered regulation specific to a particular somatic or germline cell lineage that is not detected in reconstitutions using VA13 and 293T cells. In addition, because TERT has interaction partners and biological roles that are independent of its assembly with hTR (46), it is possible that TERT variants induce disease without an impact on telomerase catalytic activity.

There are many confounding issues that complicate the interpretation of a genotype-to-phenotype correlation. Incomplete clinical and molecular work-up of many patients and patient families is a challenge that is inherent to understanding the basis of inherited human disease. For disease linked to telomere shortening in particular, one would expect symptoms to depend on the complex combination of genotype, telomere length inheritance, and the proliferative history of each tissue sensitive to telomere length for differentiation or renewal (21). In future studies it will be of interest to continue the characterization of variants in hTR, TERT, and other telomerase and telomere proteins in DC, AA, MDS, and PF and to be able to complete clinical and molecular work-up of many patients and patient families.

Structure/Function of hTR—From the perspective of the overall relationship between hTR structure and function, it is curious that most disease-associated hTR variants affect base-pairing of a stem (excepting DC-linked substitutions of the template itself). In support of the significance of disrupted stem pairing as a loss-of-function mechanism, substitutions that replace a canonical base pair with a wobble base pair (37A→G in P1 and 116C→U in P2) assemble holoenzymes with less severe functional defects than are imposed by more disruptive substitutions in the same stem (204C→G in P1, 293T→A and ΔA96→G in P2). The affected stems span the full length of hTR including the H/ACA-motif stems required for hTR accumulation and the P1, P2, P3, and P6.1 stems required for hTR–TERT interaction and catalytic activity (Fig. 1A and 5A). Pairing of each of these stems was previously shown to be critical for human or mouse telomerase holoenzyme catalytic activity monitored by TRAP (44, 47).

Considering all of the hTR substitutions examined here, our investigation reveals that not all absolutely conserved residues of vertebrate TER have sequence-specific roles in human telomerase holoenzyme assembly or catalytic activation. Some of these conserved residues, particularly at positions of the CR4/5 internal loop adjacent to P6a (Fig. 5A), may instead serve to increase the efficiency of hTR folding. Other regions of the CR4/5 internal loop have an important role in promoting hTR–TERT interaction. Furthermore, independent of hTR–TERT interaction, conserved residues of the P6.1 loop are required for telomerase activation. We suggest that as proposed for the potentially analogous ciliate TER loop IV (48), vertebrate TER P6.1 contributes to telomerase catalytic activation by allosteric regulation of TERT.

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