Functional characterization of telomerase RNA variants found in patients with hematologic disorders

Hinh Ly, Rodrigo T. Calado, Paulette Allard, Gabriela M. Baenninger, Peter M. Lansdorp, Neal S. Young, and Tristram G. Parslow

Introduction

Telomerase is a specialized DNA polymerase that adds long, repetitive stretches of simple telomeric DNA sequence (ie, [TTAGGG]n in the vertebrates) onto the chromosomal termini.\(^1\) The minimal telomerase holoenzyme is a ribonucleoprotein (RNP) complex with 2 components: a protein (called TERT) that has RNA-dependent DNA polymerase (ie, reverse transcriptase) activity, and an associated RNA (called TERC) that serves as its template.\(^2\) Vertebrate TERCs are believed to adopt a complex, folded structure due to extensive intramolecular base-pairing that forms an ordered series of paired (P) or helical regions. This phenotypically conserved structure, depicted for human TERC (hTERC) in Figure 1, is viewed as comprising 4 separate conformational domains, termed the core, CR4-CR5, box H/ACA, and CR7 domains, respectively, together with an interposed region of variable length and sequence called the hypervariable paired region.\(^2\) Site-directed mutagenesis studies of hTERC have validated much of this predicted structure and confirmed that each of the 4 conserved domains contributes features necessary for telomerase function. At certain locations, including the single-stranded templating region that is copied into telomeric DNA, specific RNA base sequences are required for biologic activity. As is true of many biologically active RNA molecules, however, most of the internally base-paired regions of hTERC can be extensively mutated without loss of function provided that the normal base-pairing pattern is preserved.\(^3,4\) Those findings, together with the strict conservation of this base-pairing pattern in evolution, imply that telomerase activity depends on specific features of both base sequence and secondary structure in hTERC, some of which have yet to be fully elucidated. The core domain includes the templating region along with a conserved pseudoknot structure that serves both to delineate the templating region and to mediate dimerization of hTERC molecules during telomerase assembly.\(^5,6\) The core and CR4-CR5 domains each contribute to binding of the TERT protein,\(^4\) though the specific requirements for binding are unknown. The box H/ACA domain is required for proper processing, stability, and trafficking of hTERC within cells, whereas the CR7 domain provides signals that target hTERC into a specialized nuclear compartment where telomerase RNP assembly occurs.\(^7-10\)

Inherited mutations in hTERC underlie one form of a rare human disorder, known as dyskeratosis congenita (DC) that involves hematopoietic failure. This disorder is characterized by abnormal skin pigmentation, nail dystrophy, and oral leukoplakia, and is often complicated by life-threatening bone marrow failure and immunodeficiency.\(^11\) The autosomal dominant form of DC results from germ line inheritance of mutations of the hTERC gene, and all affected individuals studied to date are heterozygous (ie, carry one mutant and one wild-type allele) for the gene. Lymphocytes from affected individuals show decreased hTERC expression, decreased telomerase activity, and significantly reduced telomere lengths as compared with those from age-matched controls.\(^12,13\)

Building on those findings, we and others have investigated whether telomerase mutations also contribute to other, more common disorders that involve bone marrow failure, such as...
 assent of apparently acquired forms of those disorders have revealed an
boxed H/ACA and CR7 domains. Boxed regions show a large deletion that completely removes the sequences of the
(see Figure 2A for details). Nucleotide deletions are indicated by thick lines, whereas
boxed regions show a large deletion that completely removes the sequences of the
box H/ACA and CR7 domains.

Materials and methods

Cloning of novel hTERC variants

Peripheral blood leukocytes were collected from patients with acquired AA
or essential thrombocythemia, each of whom had given informed consent
according to protocols approved by the institutional review board of the
National Heart, Lung, and Blood Institute. The hTERC gene was amplified
from genomic DNA isolated from these samples as described previously.15

Telomere length measurements

Average telomere length in peripheral blood leukocytes collected from
patients and control individuals was measured by flow-fluorescence in situ
hybridization as previously described.18,19

Engineering VA13+hTERT stable cell line

DNA encoding the wild-type hTERT gene was cloned into the plasmid
pBabe-Puro at the EcoRI and SalI restriction sites. The resulting vectors
were transfected transiently into the PA317 packaging cell line, which
produces amphotrophic murine leukemia virus (MuLV) pseudotyped par-
ticles, via the calcium phosphate transfection technique (Gibco-BRL,
Carlsbad, CA). Pseudotyped particles containing the hTERT expression
cassette were harvested 48 hours after transfection and were used to infect
VA13 (ATCC, Manassas, VA), a human lung fibroblast cell line transformed
by SV40 large T-Ag, that expresses neither the hTERT nor the hTERC
component of the human telomerase complex. Purumycin-resistant colo-
ny were selected by growth in Dulbecco modified Eagle medium supplemented
with glucose (4.5 g/L), 10% bovine calf serum, and 2.0
μg/mL puroycin. About 100 drug-resistant colonies of the VA13+hTERT
cells were pooled and expanded for use in the subsequent experiments.

In vivo reconstruction of telomerase activity

Wild-type or mutant pcDNA3-hTERC DNAs (1 μg) were transfected into
VA13+hTERT cells (at approximately 70% confluence) in 6-well polystyr-
ene dishes using SuperFect transfection reagent (Qiagen, Valencia, CA)
according to the manufacturer’s instructions. In the cases where 2 different
versions of the hTERC gene were expressed simultaneously in the
VA13+hTERT cells, the genes were cloned into the pBud-CE 4.1 vectors
(Stratagene, La Jolla, CA) to be expressed from either the cellular EF1α
promoter or the viral cytomegalovirus (CMV) promoter. Transfection
efficiency was monitored by scoring green fluorescent protein expression
under confocal microscopy in parallel transfection reactions supplemented
with the reporter vector pEGFP (Stratagene). Approximately 48 hours after
transfection, cells were scraped from the dish in the presence of 1 mL cold
phosphate-buffered saline. Cellular extracts were then prepared in 1 ×
CHAPS (3-[3-cholamidopropyl)dimethylammonio]-1-propane sulfo-
nate) lysis buffer as suggested by the manufacturer (Chemicon Interna-
tional, Temecula, CA). Telomerase activity of the cellular extract from
2 × 10^6 cells was assayed using the TRAPEze Telomerase Detection Kit
following the manufacturer’s directions (Chemicon International), except
that polymerase chain reaction (PCR) was performed as follows: 95°C for 2
minutes; 25 cycles of 94°C for 10 seconds, 50°C for 30 seconds, 72°C for
30 seconds; and 72°C for 5 minutes. Products were analyzed on a 12%
native polyacrylamide gel and examined by phosphor imaging (Molecular
Dynamics, Sunnyvale, CA).

Telomerase activity in primary cells

Peripheral blood cells collected from patients or healthy (control) individu-
als were cultured in RPMI-1640 with t-glutamine and 10% fetal calf serum
in the presence of 5 μg/mL phytohemagglutinin and 40 IU/mL interleukin-2
for 4 days at 37°C under 5% CO2. An aliquot of cells was then dually
stained with anti-CD19–fluorescein isothiocyanate (FITC) and anti-CD3–
phyceroerythrin (PE) (BD Biosciences, San Diego, CA) for analysis in an
LSRI flow cytometer (BD Biosciences). Protein extract was prepared from
2 × 10^6 cells, and serial dilutions containing 1.0 μg, 0.5 μg, and 0.1 μg

Figure 1. Schematic depiction of the predicted secondary structure of hTERC.
This schematic depiction is as proposed by Chen et al.2 The 8-base template
sequence (rectangle) and other structural features are indicated, including the core,
CR4-CR5, box H/ACA, and CR7 domains, and the hypervariable paired region.
Mutations associated with DC are indicated in red; those associated with AA, MDS, or
PNH are in green. The G228A and G58A variants (in blue) are fully active in vitro (Fu
and Collins,17 Marrone et al,27 and the present study) and also found in healthy
variants have been tested, however, the generality of these findings

In this study, we systematically examine the functional proper-
ties of 10 previously reported DC-, AA-, and MDS-associated
variants of hTERC in a cell-based telomerase reconstitution assay. We
also report here for the first time 2 new hTERC variants
identified in patients with AA or essential thrombocytemia. Our
findings indicate that most of these disease-associated hTERC
variants have significantly impaired telomerase biologic activity. We
show that most of these defects are attributable to alterations in
hTERC secondary structure, as compensatory mutations designed
to restore the normal base-pairing pattern also restore enzymatic
activity. Our data provide support for the hypothesis that mutations
in the RNA component of telomerase may cause or predispose to
these disorders in a significant subset of patients.

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Northern blotting analysis

Wild-type or mutant pcDNA3-hTERC vectors (6 μg) were transfected into VA13 + hTERT cells (at approximately 70% confluency) in 100-mm polystyrene dishes using SuperFect transfection reagent. Approximately 48 hours after transfection, Trizol reagent was used to extract total cellular RNA as suggested by the manufacturer (Invitrogen, Carlsbad, CA). Northern blot analysis was performed essentially as described.20

Immunoprecipitation Northern blotting analysis

FLAG-tagged hTERT protein was expressed in vitro from the pCR3-FLAG-hTERT vector using the TnT quick-coupled transcription-translation system (Promega, Madison, WI) in the presence of 200 ng in vitro–transcribed, gel-purified CR4-CR5 fragment from hTERC (spanning hTERC nucleotides 239 to 332) at 37°C for 2 hours. The resulting telomerase complexes were affinity-enriched on anti-FLAG agarose beads (Sigma, St Louis, MO). To detect hTERT-bound telomerase RNAs, Northern blotting was performed on the enriched telomerase preparations as described in our previous report.3

Results

Our study focused on 12 distinct hTERC variant sequences identified in recent surveys of patients with DC, AA, PHN, or MDS.15,16,21 The variants and their associated clinical and laboratory findings are summarized briefly in Table 1. Only one hTERC variant (G228A) analyzed here was observed in healthy controls as well as in patients with AA (Figure 1 and Table 1). The 11 remaining variants were each detected only in affected individuals or their relatives. The latter group includes 2 novel hTERC variants (A117C and Δ389-390) that we discovered in 2 unrelated patients; the former exhibited typical AA along with idiopathic hepatic cirrhosis, whereas the latter was diagnosed with essential thrombocytopenia. As essential thrombocytopenia is a clonal disorder, the germ line origin of the hTERC variant was established by concurrent results obtained from buccal mucosa specimens from this patient as well as from his son. All of the patients whose variant hTERC alleles were the subject of this study were heterozygous (ie, they carried one wild-type hTERC gene in addition to the variant).

Table 1. Clinical and laboratory data from patients with hTERC variants

<table>
<thead>
<tr>
<th>hTERC sequence variant</th>
<th>Clinical diagnosis</th>
<th>Telomere length</th>
<th>Telomerase activity*</th>
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</thead>
<tbody>
<tr>
<td>Core domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C116T</td>
<td>AA (severe pancytopenia)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>C204G</td>
<td>AA (moderate pancytopenia)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A117C</td>
<td>AA (severe pancytopenia)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G143A</td>
<td>DC</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Δ96-97</td>
<td>DC</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>CR4-CR5 domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G305A</td>
<td>AA (moderate)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G322A</td>
<td>MDS</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>H/ACA domain</td>
<td></td>
<td></td>
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<tr>
<td>Δ389-390</td>
<td>Essential thrombocytopenia</td>
<td>ND</td>
<td>–</td>
</tr>
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<td>C408G</td>
<td>DC</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>G450A</td>
<td>AA (severe)</td>
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<td>+</td>
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<tr>
<td>CR7 domain</td>
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<td></td>
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<tr>
<td>Δ378-451</td>
<td>DC</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Hypervariable paired region</td>
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<td></td>
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</tr>
<tr>
<td>G228A</td>
<td>AA (moderate) or healthy</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Sources for the original descriptions, associated clinical findings, and telomere length data are as follows: C116T and C204G22; G143A and Δ96-9722; A117C and Δ389-390, present study; G305A and G322A22; and C408G and Δ378-451.23 Original descriptions, associated clinical findings, and telomere length data for A117C, G305A, and G322A are from the present study. Telomere lengths of peripheral blood lymphocytes are expressed in comparison to those of age-matched healthy individuals assayed simultaneously, as reported in the indicated references: +++, within reference range; +++, 2 kb to 3 kb shorter than the reference range; +, 3 kb to 6 kb shorter than the reference range; ND, not determined.

Mutations of essential RNA sequences and structures in the CR4-CR5 domain

We next examined the properties of 2 separate sequence variants of the conserved CR4-CR5 domain. These variants, G305A and G322A, had been identified in patients with AA and MDS, respectively, and each involved separate regions of this domain.
whose structure is ambiguous. Although residue G305 was originally predicted to reside in a single-stranded region of hTERC, subsequent mutational studies of its murine orthologue (mTERC) have suggested that the corresponding residue is paired, forming part of a short helical region denoted P6.1 (Figure 1). The possible existence and function of a P6.1 helix in hTERC has not been explored in detail, however. Similarly, recent studies using chemical and enzymatic probes of RNA structure have suggested that residue G322 may pair with residue C247 as part of an elongated P5 helix in hTERC (Figure 1 and Figure 3A, line 1), but no functional evidence for such interaction has been reported.

Testing of the disease-associated G305A and G322A variants indicated that each was expressed at normal concentrations within transfected cells (Figure 2D, lanes 4 and 5) but was nevertheless functionally defective, supporting no more than 1% of wild-type hTERC activity (Figure 2B, lanes 32-37, 41-46). Compensatory mutations failed to restore activity to an appreciable degree in either instance (Figure 2B, lanes 38-40, 47-49). Because these findings appeared, in the case of G305A, to be at odds with the reported properties of the P6.1 helix in mTERC, we extended our analysis by testing additional hTERC mutants designed either to disrupt all 4 base pairs of the putative P6.1 helix simultaneously (mutants P6.1[up] and P6.1[dn]) or to replace them with alternative paired bases (mutant P6.1[comp]). Telomerase enzymatic function was completely abrogated by any of these mutations (Figure 3C, lanes 4-12), a finding that provides no information regarding the existence of a P6.1 structural counterpart in humans, but suggests that the specific base sequence in this region of the CR4-CR5 domain is critical for hTERC function.

In mTERC, integrity of the P6.1 stem structure is required for interaction with the catalytic mTERT protein. Because the requirements for human hTERT protein binding to hTERC have not been precisely mapped, we therefore asked whether mutations of the residues corresponding to P6.1 in hTERC would affect this interaction in cells. To that end, we compared telomerase RNP complexes assembled in cells expressing hTERC fragments (spanning nucleotides 239 to 332) that contained either the wild-type CR4-CR5 domain or its mutants. Telomerase RNP complexes were reconstituted in vitro using the rabbit reticulocyte lysates to express a FLAG-tagged hTERT protein in the presence of the synthetic RNA molecule containing various sequences of the CR4-CR5 domain (“Materials and methods”). Telomerase RNP complexes were then immunoprecipitated from the lysates using an anti-FLAG antibody against the FLAG-tagged hTERT protein, and were then probed for hTERT by Northern blotting. As illustrated in Figure 3D, all 3 of the engineered CR4-CR5 mutants we tested showed substantially impaired binding to hTERT protein, although the single mutant that retained a P6.1-like pattern of base-pairing bound to a modestly greater extent than those that did not. The data on the P6.1(up) and P6.1(dn) mutants are consistent with those published recently by Moriarty and colleagues. Taken together, these findings suggest that specific sequences in the CR4-CR5

Figure 2. Disease-associated mutations located in the core domain or the box H/ACA and CR7 domains abolish telomerase activity. (A) Telomerase enzymatic activities as determined in VA13-hTERT cells for naturally occurring hTERC mutations and their derivatives involving the core, box H/ACA, and CR7 domains. When the natural sequence variants are located within a paired region (eg, C116T), the nucleotides that are predicted to base-pair with them are also mutated to the complementary bases (eg, C116T[comp]). The compensatory mutations (eg, C116T[comp]) are created in order to restore the helical structures. The sequence changes are indicated in bold. Telomerase activity of each mutant is expressed in comparison to that of the wild-type (wt) (++, 20%-100%; +, 2%-20%; +, 1%-2%; −, undetectable), based on 2 or 3 independent determinations. (B, C) Representative TRAP gels showing the relative telomerase enzymatic activities obtained from the representative substitution or deletion mutations and compensatory mutations. Serial 5-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lane 54 shows a negative control composed of wild-type (WT) cell lysate denatured at 95°C for 5 minutes prior to assay. Lane 55 shows PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit. Lane 56 shows cells transfected with the pcDNA3.1 vector control lacking the hTERC coding sequence. Lane 57 shows the control vector containing the hTERC coding sequence. Lane 58 shows PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit. Lane 59 shows RNA prepared from cells that were transfected with the pcDNA3.1 vector lacking the hTERC coding sequence. Cellular α-actin mRNA (bottom) was assayed in parallel.
region of hTERC are needed for optimal hTERT protein binding, though it remains unclear whether a P6.1-like helical structure contributes to this binding as it does in the murine telomerase.

Divergent phenotypes of sequence variants in the box H/ACA domain

We tested 2 different hTERC sequence variants that map to the box H/ACA domain (Figure 1). This domain is suspected to interact with the cellular protein dyskerin and to help direct the nucleolytic processing of hTERC from a larger RNA precursor, and it is also required for subsequent stability and localization of hTERC within cells.12 When tested in our assay, one of these informative variants. Serial fivefold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lanes 16 and 27 of panel B and lane 13 of panel C indicate negative controls in which the wild-type cell lysates were denatured at 95°C for 5 minutes before analysis. Lanes 17 of panel B and 14 of panel C show PCR products amplified from the control TSR8 DNA template supplied in the kit. Lanes 18 of panel B and 15 of panel C show cells transfected with the pcDNA3.1 vector lacking the hTERC coding sequence. (D) Northern blotting analysis of affinity-enriched telomerase complexes assembled in vitro using wild-type P6.1 stem (spanning TERC nucleotides 239 to 332) or its mutants. Telomerase RNA-protein complexes were first assembled in the rabbit reticulocyte lysates. The negative control (lane 4) was a lysate that received no hTERT expression vector.

Figure 3. Mutations of the hypervariable region or the CR4-CR5 or box H/ACA domains show variable degrees of telomerase activity. (A) Telomerase enzymatic activity in VA13 + hTERT cells expressing various hTERC variants involving the hypervariable paired region or the CR4-CR5 or box H/ACA domains. Natural sequence variants and engineered mutations are indicated in bold. The telomerase activity for each variant is expressed in comparison to that of the wild-type (+ +++, 20%-100%; + +, 2%-20%; +, 1%-2%; –, undetectable) based on 2 or 3 independent determinations. (B, C) Representative gels showing the relative telomerase enzymatic activities obtained from informative variants. Serial fivefold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lanes 16 and 27 of panel B and lane 13 of panel C indicate negative controls in which the wild-type cell lysates were denatured at 95°C for 5 minutes before analysis. Lanes 17 of panel B and 14 of panel C show PCR products amplified from the control TSR8 DNA template supplied in the kit. Lanes 18 of panel B and 15 of panel C show cells transfected with the pcDNA3.1 vector lacking the hTERC coding sequence. (D) Northern blotting analysis of affinity-enriched telomerase complexes assembled in vitro using wild-type P6.1 stem (spanning TERC nucleotides 239 to 332) or its mutants. Telomerase RNA-protein complexes were first assembled in the rabbit reticulocyte lysates. The negative control (lane 4) was a lysate that received no hTERT expression vector.

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A functionally intact sequence variant of the hypervariable paired region

We examined one additional natural variant hTERC with a point substitution (G228A) in the 5′ strand of the hypervariable paired region (Figure 1), a region whose base sequence has diverged...
A lack of dominant-negative effect in cells simultaneously expressing both the wild-type hTERC gene and the individual sequence variants

Since previous studies have shown that human telomerase enzyme functions as a dimeric or multimeric complex, consisting of at least 2 hTERC RNAs and 2 hTERT proteins, it is possible that some of the disease-associated hTERC sequence variants may act as dominant-negatives to prevent the proper formation of a functional telomerase RNP. To test this possibility, we forced the expression of 2 different versions of the hTERC gene simultaneously in the same cell by expressing both genes from the same vector (Figure 4). Vectors that expressed either 2 wild-type copies of hTERC or a wild-type and the mutated copy of the gene were transfected into the VA13+hTERC cells, and cell lysates were prepared for the TRAP assay as outlined above. No major differences were observed in samples that expressed the individual mutated hTERC variants together with the wild-type copy as compared with the samples that expressed the wild-type hTERC copies (Figure 4), regardless of the promoters from which the genes were being expressed (Figure 4A).

Figure 4. Telomerase enzymatic activity in VA13+hTERT cells simultaneously expressing various hTERC variants and the wild-type copy from a single pBud-CE 4.1 vector series. (A) While the wild-type hTERC copies are expressed from both the cellular EF1α and the viral CVM promoters, the mutated hTERC genes (ie, either the G305A or the G322A) are alternatively expressed from either the EF1α or the CMV promoter with respect to the wild-type hTERC copy. Serial 5-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. (+) Telomerase function of an aliquot of the sample that expressed both wild-type copies was inactivated by denaturing the sample at 95°C for 3 minutes prior to assay. (–) Telomerase function of an aliquot of the sample that expressed the wild-type hTERC copies (Figure 4), regardless of the promoters from which the genes were being expressed (Figure 4A). Similar observations were made for the 4 different disease-related hTERC variants described in our previous study (Figures 4D and 1). Taken together, these data suggest that the disease-associated hTERC sequence variants tested thus far do not function as dominant-negatives in human cells.

Telomerase enzymatic function of primary cells collected from patients

To determine whether haploinsufficiency can also be observed directly in primary cells, we analyzed the effect of the hTERC mutations in the T lymphocytes collected from either a healthy individual or the patients with AA who carry either the A117C or the C204G that were available to us. By using an equal amount of the protein extracts prepared from the primary cells, which have been expanded in tissue cultures, we compared the ability of the extracts to add telomeric repeats into a DNA substrate using the TRAPeze kit as described above. Compared with the sample of a healthy individual, both samples collected from the patients with AA showed moderately reduced telomerase enzymatic function (Figure 5), which is compatible with what might be expected in cells with one wild-type and one inactive hTERC allele. These data confirm the observations that were made with the transient reconstitution of the telomerase RNPs in the VA13+hTERT cells for the corresponding hTERC sequence alterations (Figure 4), further emphasizing the haploinsufficiency effect of the mutations and the validity of our in vivo reconstitution study.
Discussion

This study provides functional evidence that variant telomerase RNA alleles found in some patients with bone marrow failure are unable to support normal levels of telomerase enzymatic activity. Together with earlier reports,3,13,17 our data are consistent with the hypothesis that inherited defects in telomerase function and telomere maintenance contribute to the pathogenesis of such disorders in a subset of patients, even when no familial tendency is apparent. The levels of telomerase enzymatic activity supported by individual disease-associated hTERC variants in the cell-based assay we used correlated very well with independent measurements of telomere lengths in lymphocytes from patients who carried those variant alleles (Table 1). In particular, telomeres in patients harboring the C116T, A117C, C204G, or G305A variants have previously been reported to be markedly shorter (averaging 3.7-4.6 kb) than those of healthy age-matched individuals (8-12 kb),15 and none of these 4 variants proved able to support detectable telomerase activity in our cell-based vivo telomerase reconstitution assay. Primary cells harvested from representative patients who were heterozygous for either the A117C or the C204G allele, moreover, showed correspondingly lower levels of telomerase activity (Figures 2B, 3B, and 4). Conversely, the normal telomerase activities of the G228A and G450A variants are consistent with the essentially wild-type average telomere lengths observed in individuals heterozygous for these alleles, some of whom are hematologically normal.15 These correlations help support the validity of the assay we employed in this study, and strengthen the view that the telomere shortening seen in some patients with bone marrow failure may result directly from telomerase dysfunction.

The naturally occurring mutations observed in this study are distributed throughout the hTERC molecule. Their effects confirm and extend earlier findings from site-directed mutagenesis, indicating that all 4 domains of hTERC contribute to function and that the intricately base-paired structure of this RNA is critical for its biologic activity.3,5,34,35 Indeed, it is striking that a high proportion of the seemingly minor point mutations can severely compromise telomerase function by perturbing RNA structure. These include 3 adjacent mutations in the P2b stem, a single point substitution in the P2a.1 stem, and another point substitution located within the P1 stem, all of which are involved in the proper folding of the pseudoknot in the hTERC core domain. Our finding that compensatory mutations can restore activity to most of these hTERC pseudoknot variants, coupled with evidence from structural analyses using NMR spectroscopy,28,34 highlights the importance of the normal base-pairing pattern in this region of the RNA. It remains to be determined whether this reflects a role of the pseudoknot in global folding of the hTERC monomer, in dimerization of hTERC, or in some other aspect of telomerase holoenzyme assembly and function. While this manuscript was in preparation, a similar study appeared,27 which provided a comprehensive functional analysis of 4 disease-associated mutations (i.e., C408G, G228A, Δ96-97, Δ378-451) that overlapped with some of those analyzed in our current work. This study also suggests that optimal telomerase function depends on the proper folding of the hTERC RNA molecule.27

Our finding that one of 2 variants in the box H/ACA domain was functionally defective is in full accord with results from earlier studies that used site-directed mutagenesis,12,17,27 though we de-

Acknowledgment

Xiaoying Yang is acknowledged for excellent technical assistance.

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References

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