The reverse transcriptase telomerase copies an internal RNA template to synthesize telomeric simple-sequence repeats. In the cellular context, telomerase must elongate its few intended substrates (authentic chromosome ends) without spurious activity on other potential substrates (chromosome ends created by damage, repair, or recombination). Many mechanisms have been proposed to account for the biological substrate specificity of telomerase, with most models focusing on protein-protein interactions between telomerase and telomeric chromatin. Telomerase activity assays testing the elongation of model oligonucleotide substrates have revealed that in addition to hybridization with the RNA template, optimal DNA substrates also engage telomerase protein-based interaction sites. The physiological significance of these non-template interaction sites has not been established. We used in vitro reconstitution to assemble telomerase enzymes with variant telomerase reverse transcriptase proteins. Several telomerase enzyme variants retained a wild-type level of catalytic function in vitro when assayed using an artificial sequence substrate but exhibited reduced activity on a more physiological telomeric-sequence substrate. Telomerase that demonstrated this defect in telomeric substrate usage in vitro also failed to support telomere length maintenance in vivo. Our findings suggest that non-template interactions of the telomerase ribonucleoprotein with telomeric DNA play a critical role in supporting telomerase function on its appropriate cellular substrates.

Most eukaryotic cells contain a single essential reverse transcriptase (RT). 1 This telomerase ribonucleoprotein (RNP) RT replenishes the terminal sequences of linear chromosome ends that are lost with each round of genome replication (reviewed in Ref. 1). Telomerase activity can be reconstituted in vitro with only two enzyme components, the telomerase reverse transcriptase protein (TERT) and telomerase RNA (reviewed in Refs. 2 and 3). These two evolutionarily conserved subunits and additional holoenzyme proteins are required for telomerase function in the cell. Telomerase is specialized in comparison with viral RT enzymes in two particularly notable ways. First, both in vitro and in vivo, the telomerase active site copies only a precisely bounded template within the telomerase RNA (4, 5). Secondly, as is evident from in vitro assays, primer substrate specificity is determined by hybridization to the RNA template and also by interaction with other single-stranded DNA binding sites in the telomerase RNP (reviewed in Ref. 6).

To date, it has been difficult to identify which proteins or protein regions provide non-template DNA interaction sites within a telomerase holoenzyme complex. The existence of these “anchor” and “nascent product” binding sites has been inferred from the preferential elongation of in vitro substrates with specific sequence contents in their non-template hybridizing, 5’ regions (e.g. Refs. 7–12). TERT itself can be cross-linked to site-specific locations in a primer 5’ region, in a manner specific for telomeric sequence and dependent on primer 3’ end alignment with the template (13). 2 However, TERT cross-link contacts do not necessarily represent the most functionally critical motifs conferring sequence-specific substrate interaction. In fact, in ciliate, yeast, and human telomerase reconstitution experiments, telomerase enzymes lacking some or all holoenzyme proteins have altered substrate binding and elongation properties when compared with wild-type holoenzyme (10, 14–16). These studies strongly suggest that the holoenzyme context influences the substrate interaction properties of telomerase RNP.

For human telomerase, DNA interaction with protein-based binding sites is the predominant mechanism governing substrate preference in vitro (12). However, the physiological relevance of this interaction for telomere length maintenance has not been established: DNA hybridization with the RNA template or protein-protein interactions could be sufficient to recruit and position telomerase RNP at a chromosome end. Consistent with the idea that protein-protein interactions between telomerase and telomeric chromatin play an important regulatory role, in vitro assays have detected normal telomerase activity in extracts of yeast and human cells that nevertheless exhibit an inhibition of telomerase activity at telomeres (17–19). This same discrepancy has been observed upon in vivo reconstitution of some yeast and human TERT sequence variants (20–23). For human TERT (hTERT), substitution of wild-type sequence with the hexapeptide NAAIRS within an N-terminal 65 amino acid region or at the extreme C terminus produces a phenotype termed DAT, for “dissociation of activities of telomerase” in vitro and in vivo (22, 23). These and related studies (24, 25) have been interpreted to suggest that DAT regions of hTERT and associated holoenzyme proteins mediate the physical localization of telomerase RNP to telo-

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1 The abbreviations used are: RT, reverse transcriptase; RNP, ribonucleoprotein; TERT, telomerase reverse transcriptase; hTERT, human TERT; DAT, dissociation of activities of telomerase; TRAP, telomeric repeat amplification protocol; hnRNP, heterogeneous ribonucleoprotein; TRF2, telomere repeat binding factor 2.

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meres, presumably regulating telomerase activity by regulating enzyme recruitment to telomeric chromatin.

Direct protein-DNA interaction could also be envisioned as a key determinant of either telomerase physical association with telomeric chromatin (described as “recruitment”) or in the accurate positioning of telomerase at an extreme chromosome 3’ terminus (termed “end-positioning”). Here we show that hTERT DAT variants reconstitute telomerase RNPs that are inhibited in the elongation of telomeric sequence substrate in vitro. Because these variant telomerases exhibit activity comparable with wild-type enzyme when a more artificial sequence substrate capable of template hybridization is used, we suggest that the compromised interaction of single-strand telomeric repeat DNA with telomerase RNP at non-template DNA binding sites prevents DAT-variant telomerase function in telomere length maintenance.

EXPERIMENTAL PROCEDURES

Cell Lines and Extracts—Retroviral infections, selection in hygromycin B, cell growth, and telomere length analysis were performed as described previously (26). Whole cell extracts were made by freeze-thaw lysis. Cells were resuspended in hypotonic buffer (20 mM HEPES, pH 8.0, 2 mM MgCl₂, 0.2 mM EGTA, 10% glycerol, 1 mM dithiothreitol) and subjected to 3 cycles of rapid quick-freeze and thaw at 37°C. Lysate was supplemented to 0.4 M NaCl and clarified by centrifugation in a microfuge at maximum speed for 15 min at 4°C. Extracts were adjusted to 0.2 M NaCl and then quick-frozen in liquid nitrogen. Immunoprecipitation shown in Fig. 4 was performed using monoclonal antibodies pre-bound to protein G-Sepharose, with final buffer conditions of 20 mM HEPES, pH 8.0, 50 mM NaCl, 5 mM MgCl₂, 20% glycerol, 0.01% Nonidet P-40, and 2 mM dithiothreitol. Samples were bound for 1 h at 4°C and were washed in the same buffer.

Telomerase Activity Assays by TRAP—A modified version of the original TRAP assay was performed as described previously (27). Briefly, extracts were diluted 10-fold into TRAP assay buffer and incubated with primer M2 (AAATCCGCTGAGCCAGAGTT) or SM2 (AAATCGTCGACGACGAA). Telomerase was heat-denatured before addition of reverse primer CX3 (CGGGCCGCTAACCCTAACCCTA) and PCR amplification. Products were analyzed by electrophoresis using 10% acrylamide non-denaturing gels. For maximal sensitivity to non-specific inegration, we used an internal control (IC) for the PCR amplification step that is larger than the telomerase products.

Telomerase Activity Assays by Direct Primer Extension—Whole cell extracts were diluted 5-fold into an activity assay buffer with final composition of 50 mM Tris acetate, pH 8.0, 50 mM KCl, 1 mM MgCl₂, 5 mM β-mercaptoethanol, 1 μM telomeric repeat primer (G₁₀T₆), 0.5 mM TTP and dATP, 5 μM unlabeled dGTP, and 2.5 μM (α³²P)dGTP (800 Ci/mmol, PerkinElmer Life Sciences). Reactions were incubated at 30°C for 60 min. Product DNA was analyzed by electrophoresis using 9% acrylamide denaturing gels.

RESULTS

DAT Phenotype hTERTs Are Catalytically Active yet Defective in Telomere Length Maintenance in Non-transformed Primary Cells—Studies characterizing the hTERT DAT phenotype by Armbruster et al. (22) and Banik et al. (23) employed the HA5 cell line. HA5 human embryonic kidney cells are mortal but are transformed by expression of a region of simian virus 40 encoding the large T and small t antigens (28). To examine whether the DAT phenotype is contingent upon an oncogene-mediated regulation of telomerase access to telomeres (26), we independently expressed wild-type hTERT or one of three different DAT hTERTs in non-transformed primary cell lines. We tested DAT hTERT variants containing NAIRIS substitution within the N-terminal DAT region, substituting amino acids 92–97 (N-DAT92) or amino acids 122–127 (N-DAT122), or at the extreme C terminus, substituting amino acids 1127–1132 (CDAT1127). Stable expression of N-terminally green fluorescent protein-tagged wild-type hTERT, the two N-DAT hTERTs and C-DAT hTERT (Fig. 1A) was directed by integration of pBABI retroviral expression vectors into both IMR90 primary lung fibroblasts and GM01787 primary dermal fibroblasts. The resulting lines produced comparable levels of tagged wild-type and variant hTERTs as assessed by immunoblot using green fluorescent protein polyclonal antiserum (data not shown). As reported previously for HA5 cell lines (22, 23), extracts from the non-transformed primary cell lines expressing hTERT demonstrated robust, roughly comparable levels of telomerase activity when examined using the TRAP assay (Fig. 1, B and C). Parental cell lines or cell cultures selected for expression of retroviral vector without hTERT lacked telomerase activity detectable by TRAP using whole cell extract (Fig. 1, B and C). The restricted S-phase induction of endogenous TERT involved in telomere capping (29) is likely to be unaffected in the cell lines generated in this study, because the recombinant hTERT expression constructs produce a low physiological level of TERT accumulation (26) instead of the high over-expression level required to out-compete endogenous RNP assembly.

Telomeric restriction fragment length in all cell lines was evaluated over the course of post-selection population doublings by in-gel Southern hybridization with a telomeric repeat oligonucleotide. Data for the five GM01787 cell lines are shown in Fig. 1D; identical results were obtained for the five IMR90 cell lines (data not shown). Telomere loss occurred with proliferation in the parental primary cell line (Fig. 1D, lanes 1–3) and was prevented by expression of wild-type hTERT (lanes 4–6), as telomere length and telomere hybridization signal increased dramatically upon wild-type hTERT expression. All N-DAT and C-DAT hTERT cell lines continued to experience telomere loss with proliferation (lanes 7–15). These findings reveal that the DAT phenotype is recapitulated in primary cell lines without a requirement for tumor virus transformation, although telomerase RNPs reconstituted in vivo with DAT hTERT variants exhibit levels of telomerase activity comparable with wild-type telomerase RNP in cell extract, telomere length maintenance with cell proliferation is inhibited. DAT Substitutions Compromise Telomeric Repeat Primer Elongation in Vitro—Telomerase activity assays using mammalian cell extract frequently employ the two-step TRAP assay (30) instead of the “conventional” direct primer elongation assay (31) due to the enhanced sensitivity provided by PCR amplification of the telomerase reaction products. A second difference between TRAP and conventional assays is the nature of the substrate. Although conventional assays typically employ a DNA primer with physiological telomeric sequence (e.g. permutations of T₆AG₃ repeats with the human enzyme), the TRAP assay uses an artificial, non-telomeric substrate (called M2) so that reaction products can be amplified to great abundance by PCR with distinct forward (non-telomeric) and reverse (telomeric) primers. We speculated that the hTERT DAT phenotype could reflect a compromised TERT-DNA interaction specific for telomeric sequence substrates. In this case, DAT-variant telomerase RNPs might reveal their underlying defect in vitro as well as in vivo, if assayed under appropriate conditions.

To test this hypothesis, we assayed cell extracts by TRAP (Fig. 1, B and C) and concurrently by conventional assay with the human telomeric repeat oligonucleotide (G₁₀T₆A₈) (Fig. 2, A and B). Extracts from GM01787 (Fig. 2A) and IMR90 (Fig. 2B) cell lines were assayed in duplicate; results were reproduced for both cell lines using extracts of cells harvested at different population doublings before senescence. Conventional assays performed with extracts from wild-type hTERT cell lines produced a ladder of product DNAs representing processive repeat synthesis (lanes 3–4), whereas extracts of the parental cell lines produced an irregular background labeling (lanes 1–2). Strikingly, all of the hTERT DAT variants reconstituted telo-
merase RNPs that were defective in telomeric repeat primer
elongation relative to the wild-type TERT RNP (lanes 5–10).
The extent of the defect was highly reproducible for each
hTERT DAT variant independently, but substantial differ-
ences were observed between DAT variants. The N-DAT122
hTERT telomerase RNP generated less product overall and a
reduced average product length than the wild-type telomerase
RNP, but its activity remained detectable in extracts of both
GM01787 and IMR90 lines in all assays that we conducted. In
contrast, the N-DAT92 and C-DAT1127 hTERT variants recon-
stituted telomerase RNPs with little or no detectable activity in
conventional assays, even when the same extracts prepared
and assayed in parallel by TRAP yielded activity comparable
with wild-type.

C-DAT Substitution Increases Primer Kₘ with an Entirely
Non-telomeric Primer and Reduces Telomerase Association
with hnRNP C1/C2—The relatively low telomerase RNP accu-
mulation levels in primary cells limit conventional assay sen-
sitivity. We therefore attempted to determine whether defects
evident for DAT hTERT telomerase RNPs in the conventional
assay could be studied further using a variation of the TRAP
assay. For example, we compared wild-type and DAT hTERT
extracts using different concentrations of the M2 forward
primer. Based on the potential appearance of reduced repeat
addition processivity in conventional assays with N-DAT122
RNP (Fig. 2), we also tried reverse primer combinations devel-
op to more accurately capture the initial telomerase product
length profile (32). In all of these attempts with M2 as sub-
strate, no defect was observed for DAT hTERT telomerase
RNPs in any assay (data not shown). The M2 primer is pre-
dominantly non-telomeric, derived from a site of aberrant chro-
mosome healing on human chromosome 16 (33). However,
three nucleotides at the primer 3′ end can hybridize to the
beginning of the RNA template (Fig. 3A). Because the non-
telomeric M2 primer 5′ end would make less than optimal
interactions with the non-template binding sites of wild-type
telomerase RNP, template hybridization may provide the pre-
dominant affinity for M2 substrate binding. As a result, any
DAT-variant defects due to altered substrate interaction with
non-template binding sites could be obscured in activity assays
employing M2.

A shortened version of M2 has been described differing only
by removal of the three 3′ nucleotides (Ref. 15; primer SM2,
Fig. 3A). We tested this primer in TRAP assays with titrations
of cell extract (data not shown) or with a fixed amount of
extract titrating SM2 concentration in the telomerase reaction
while maintaining constant SM2 concentration in the subse-
quent PCR (Fig. 3B). The DAT hTERT telomerase RNPs most
severely inhibited in the conventional assay (N-DAT92 and
C-DAT1127) were also the least active when assayed by TRAP.

Fig. 1. The hTERT DAT substitutions uncouple catalytic activity in vitro and telomere length maintenance in vivo. A, in this
schematic of expressed hTERT variants, a black bar within hTERT sequence represents the site of amino acid substitution with the peptide
sequence NAARL. B–C, extracts from the parental cell line and cell lines expressing the indicated hTERT variants were assayed by TRAP, using
serial 4-fold steps of extract dilution normalized by total protein (a range of 1 μg–16 ng). IC, internal control for PCR amplification. D, 2 μg of
genomic DNA from each of the indicated GM01787 cell lines harvested at the indicated population doubling (PD) post-selection was digested with
RsaI and HindIII then analyzed by in-gel Southern hybridization with a radiolabeled telomeric repeat probe. Unequal recovery of DNA from the
limited sample quantities resulted in some lane-to-lane variation of hybridization signal intensity; therefore, telomere length rather than overall
telomere signal intensity should be compared.
using the SM2 primer. The reduced activity of N-DAT92 and C-DAT1127 telomerase RNPs in SM2 TRAP assays was consistently observed across different extract preparations from both IMR90 (Fig. 3B) and GM01787 (data not shown), whether extracts were normalized by total protein or by M2 TRAP assays performed in parallel. In contrast, the activity of N-DAT122 telomerase RNP was not distinguishable from wild-type enzyme under any SM2 assay conditions examined, suggesting assays with SM2 (Fig. 3) are not as sensitive to the DAT defect as assays with telomeric repeat primer (Fig. 2). This difference could derive from a more productive coordination of template and non-template sites in the wild-type enzyme when engaged with a telomeric sequence substrate compared with a non-telomeric sequence substrate.
Previous assays have not resolved distinct phenotypes resulting from DAT substitutions at the C terminus of hTERT versus within the ~65 amino acid, N-terminal DAT “domain.” In the course of our studies, we identified two biochemical features that distinguish the C-DAT telomerase RNP from the two N-DAT telomerase RNPs examined. First, only the C-DAT telomerase RNP displayed a reduced apparent affinity for entirely non-telomeric sequence primer (SM2). This is evident when comparing activity dependence on SM2 primer concentration (Fig. 3B). For wild-type and N-DAT telomerase RNPs, activity saturated well before the maximal SM2 concentration tested (lanes 1–18). In contrast, activity of the C-DAT telomerase RNP increased with increasing SM2 concentration even to the maximal SM2 concentration tested of 0.4 μM (lanes 19–24). Secondly, C-DAT telomerase RNP activity was less efficiently immunoprecipitated using antibody against hnRNP C1/C2 (compare Fig. 4, A and B, lanes 7–9) when normalized by input activity (lanes 1–3) or by immunoprecipitation using antibody against hnRNP A1 (lanes 4–6). For example, note that although activity in the hnRNP A1 immunopurified material becomes barely detectable for both wild-type and C-DAT samples by the third dilution (lane 6), activity in the hnRNP C1/C2 immunopurified material remains detectable for the wild-type sample even to the final dilution (Fig. 4A, lane 9) but is barely detectable in the C-DAT sample by the second dilution (Fig. 4B, lane 8). This finding parallels the previous description of reduced hnRNP C1/C2 association with telomerase holoenzyme reconstituted with hTERT bearing a C-terminal tag (34). Various hnRNP proteins have been demonstrated to associate with the mammalian telomerase holoenzyme and/or to influence mammalian telomere length (reviewed in Ref. 35). Thus, reduced association with hnRNP C1/C2 could be directly responsible for the C-DAT telomerase RNP defect in substrate affinity. We conclude that the hTERT N terminus and C terminus appear to have distinct roles in establishing the properties of substrate elongation by the telomerase holoenzyme.

**DISCUSSION**

Our studies reveal that in vivo reconstitution of N-DAT and C-DAT hTERT variants produces telomerase enzymes that are defective in the elongation of a telomeric sequence primer. For a subset of the DAT variants, defects were also observed in the elongation of a non-telomeric substrate that relies exclusively on protein-based, non-template interactions for initial binding to telomerase RNP. All DAT variant telomerase RNPs failed to maintain telomere length in two non-transformed primary cell lines. Our findings demonstrate that the hTERT amino acids substituted by the hexapeptide NAAIRS in this study have important roles in determining DNA substrate usage by telomerase holoenzyme. In addition, our results suggest that the direct interaction of DNA substrate with protein-based sites in telomerase RNP contributes significantly to telomere elongation.

The mechanism by which the N-DAT and C-DAT regions of hTERT influence non-template substrate interactions remains speculative. In one plausible model, the TERT N terminus containing the N-DAT region could directly provide affinity or specificity for interaction with telomeric DNA. Alternatively, instead of forming the binding site itself, the N-DAT region could be important for efficient coordination of substrate 3’ hybridization at the template with substrate 5’ interactions at non-template anchor site(s). The hTERT C terminus could con-
tact the N-terminal domain, contributing functions similar to those described above. Alternately, protein side chains at the TERT C terminus could position a protein that provides anchor site contacts with telomeric DNA, such as hnRNP C1/C2.

A recent report describes rescue of hTERT N-DAT128 function by its fusion to the telomere repeat binding factor 2 (TRF2) (25). Although fusion of this N-DAT hTERT to TRF2 did allow partial recovery of telomere length maintenance, it did not achieve the same rate of cell growth or the full level of telomere length maintenance obtained by fusion of wild-type hTERT to TRF2. In addition, only hTERT amino acid substitutions in the C-terminal portion of the N-DAT region recovered detectable telomere length maintenance activity when fused to TRF2. This is in striking correlation with our in vitro activity assay results: hTERT N-DAT122 but not hTERT N-DAT92 retained detectable activity on a telomeric sequence primer (Fig. 2). We propose that instead of rescuing the physical recruitment of DAT-variant telomerase RNPs to telomeric chromatin, fusion to TRF2 decreased the threshold of sequence-specific telomerase RNP-DNA interaction required for recruitment or for proper end-positioning. Our findings suggest that the DAT phenotype reflects defects in different aspects of enzyme function inherent to the telomerase RNP itself. These defects include an altered interaction with DNA and compromised elongation of authentic telomeric repeat substrates.

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Human Telomerase Reverse Transcriptase Motifs Required for Elongation of a Telomeric Substrate

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