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The 5′ Guanosine Tracts of Human Telomerase RNA Are Recognized by the G-Quadruplex Binding Domain of the RNA Helicase DHX36 and Function To Increase RNA Accumulation▽

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Telomerase promotes telomere maintenance by copying a template within its integral RNA subunit to elongate chromosome ends with new telomeric repeats. Motifs have been defined within the telomerase RNA that contribute to mature RNA accumulation, holoenzyme catalytic activity, or enzyme recruitment to telomeres. Here, we describe a motif of human telomerase RNA (hTR), not previously characterized in a cellular context, comprised of several guanosine tracts near the RNA 5′ end. These guanosine tracts together are recognized by the DEXH box RNA helicase DHX36. The helicase domain of DHX36 does not mediate hTR binding; instead, hTR interacts with the N-terminal accessory domain of DHX36 known to bind specifically to the parallel-strand G-quadruplex substrates resolved by the helicase domain. The steady-state level of DHX36-hTR interaction is low, but hTR guanosine tract substitutions substantially reduce mature hTR accumulation and thereby reduce telomere maintenance. These findings suggest that G-quadruplex formation in the hTR precursor improves the escape of immature RNP from degradation, but subsequently the G-quadruplex may be resolved in favor of a longer terminal stem. We conclude that G-quadruplex formation within hTR can stimulate telomerase-mediated telomere maintenance.

Telomerase is an RNP reverse transcriptase that extends the ends of eukaryotic chromosomes by new telomeric repeat synthesis (2, 3). Enzyme activity requires the two universally conserved subunits of a functional telomerase RNP: the telomerase RNA and telomerase reverse transcriptase protein (TERT). Other proteins associate with telomerase RNA and/or TERT to promote their cellular accumulation and association or to engage the biologically active telomerase holoenzyme with telomere substrates (9, 34). In multicellular eukaryotes, somatic cells downregulate telomerase-mediated telomere maintenance as a tumor suppression mechanism (33). The progressive telomere attrition evident in most human somatic cell lineages with cell division cumulatively increases the likelihood of telomere unmasking as a signal for the DNA damage response (29). Telomerase activation is critical for long-term cellular proliferation, as reflected in the near-universal increase of telomerase subunit expression and activity in immortalized cell lines and cancers (20).

Phylogenetic sequence comparisons, directed mutagenesis, and studies of disease-linked mutations have uncovered a complexity of sequence requirements for human telomerase RNA (hTR) folding, processing, and protein interactions (5, 10, 31). As a nascent transcript of RNA polymerase II, the hTR precursor recruits two sets of the H/ACA motif RNA binding proteins dyskerin, NHP2, and NOP10 in a chaperoned multistep process culminating in the exchange of RNP biogenesis factors for the fourth stably associated H/ACA motif RNP protein, GAR1 (13, 18). Proper assembly of the hTR H/ACA motif with dyskerin, NHP2, and NOP10 is essential for precursor maturation and produces the biologically stable telomerase RNP (10). The RNA binding protein WDR79/TCAB1 can bind to the loop of the hTR 3′ stem and through this interaction increase the retention of hTR in Cajal bodies (12, 13, 23, 36, 38). Two regions within hTR separated by the 5′ stem of the H/ACA motif (Fig. 1A) are necessary and sufficient for TERT binding and RNP activity (27). In addition to the motifs described above that promote hTR function in telomere maintenance, other hTR motifs provide opportunities for negative regulation (10, 18).

DHX36 (also known as RHAU and G4R1) is a DEXH box RNA helicase independently discovered as a mediator of AU-rich element mRNA degradation and as a resolvase for G-quadruplex DNA *in vitro* (35, 37). Subsequent studies expanded the function of DHX36 to include more global roles in regulating mRNA expression and to include resolvase activity on a model RNA as well as a DNA quadruplex of parallel-strand orientation (11, 22, 25). A previous mass spectrometry analysis of human telomerase holoenzyme affinity purified by tagged TERT (18) unreliably identified DHX36 and several other helicase domain proteins (unpublished results), with the inconclusive identifications based on only a single peptide sequence (in the case of DHX36) or peptide detection in parallel mock purifications from cells lacking tagged TERT (in the case of RuvBL1 and RuvBL2, the Unigene designations for proteins also known as TIP49 or pontin and TIP48 or reptin, respectively). Helicase domain proteins participate broadly in RNA processing, RNP biogenesis, and RNP regulation with strand-separating, strand-annealing, and RNP-remodeling activities potentially relevant to telomerase RNP maturation and function (24). DHX36 in particular has several potentially relevant substrates, because model oligonucleotides with the sequence of human telomeric DNA, telomeric RNA, or the 5′ region of hTR can form Hoogsteen-base-paired G-quadruplex

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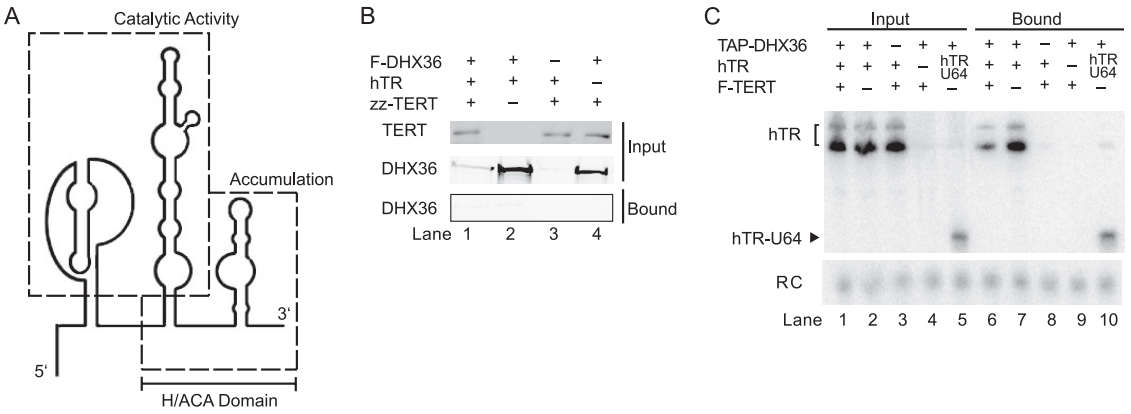

FIG. 1. DHX36 associates with hTR independent of TERT. (A) Schematic of hTR structural domains and their roles. (B) Extracts from transfected 293T cells were enriched for zz-TERT using IgG-agarose and assayed for DHX36 copurification by FLAG antibody immunoblotting. (C) Extracts from transfected 293T cells were enriched for TAP-DHX36 using IgG agarose and assayed for hTR copurification by blot hybridization. RC, recovery control. Note that lane 6 bound hTR is less than lane 7 bound hTR due to a reproducibly lower level of DHX36 accumulation when it was coexpressed with TERT, as evident in panel B.

structures *in vitro* (4, 8, 19, 26, 30, 41). We therefore investigated whether DHX36 associates with human telomerase and, if so, whether the interaction has defined sequence requirements or a more chaperone-like general specificity.

Here, we demonstrate that guanosine tracts clustered near the hTR 5′ end can be recognized by DHX36 and in particular by the DHX36 N-terminal domain specific for binding to G-quadruplex structures *in vitro* (25). Curiously, substitutions within the hTR guanosine tracts substantially reduce mature RNA accumulation. Variants of hTR disrupted for G-quadruplex formation still support robust telomerase holoenzyme catalytic activity and high repeat addition processivity. However, a reduced level of steady-state accumulation compromises their function in telomere maintenance. Together, these findings uncover a stimulatory role for G-quadruplex formation in promoting the biological function of human telomerase.

MATERIALS AND METHODS

Cell lines and constructs. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, penicillin, and streptomycin. Transient transfection of 293T cells with DNA expression constructs was performed with calcium phosphate as previously described (16). Whole-cell extract was prepared 48 h after transfection by freeze-thaw cell lysis as previously described (28). The 1774TERT cell line, created by ectopic TERT expression in primary fibroblasts from an X-linked dyskeratosis congenita patient (40), and HT-1080 cells were stably transduced by retroviral infection as previously described (40). Constructs for hTR and hTR-U64 expression from the U3 promoter were in a pBS vector backbone (16); all other transiently transfected constructs were in the pcDNA 3.1 (+) vector backbone, and retrovirally transduced constructs were in the pBABEpuro vector backbone. Proteins were N-terminally tagged with three FLAG peptides, two tandem protein A domains (zz), or a TAP tag composed of tandem protein A domains, a protease cleavage site, and a calmodulin-binding peptide.

Purification and detection of RNA and protein. For tagged protein purifications, clarified whole-cell extract corresponding to 0.5 to 1.0 mg total protein was added to tubes with 10 μl rabbit IgG-agarose (Sigma) or FLAG M2 antibody resin (Sigma) prewashed three times for 5 min in IP buffer (10 mM HEPES at pH 8, 10% glycerol, 150 mM NaCl, 0.1% NP-40). Samples were incubated at room temperature for 2 h with rotation, and then beads were washed three times for 5 min in wash buffer (IP buffer with added 0.4% NP-40 and 0.1% Triton X-100). RNA was extracted by TRIzol according to the manufacturer's protocol (Invitrogen), and a 5′-end-labeled probe complementary to hTR nucleotides 51 to 72 was used for blot hybridization as previously described (28). An *in vitro* transcript of mature hTR nucleotides 1 to 160 was added before RNA extraction from bound samples for use as a recovery control (RC). The input RNA loading control (LC) is an endogenous RNA that cross-hybridizes to the hTR probe (18). For all RNA inputs of samples used for immunopurifications, the amount loaded corresponds to 10% of the total.

For hTR detection in total cellular RNA of retrovirally transduced cell lines, 2 μg or 10 μg RNA was used for 1774TERT or HT1080 cell lines, respectively. Tagged proteins were detected with rabbit IgG (for TAP- or zz-tagged proteins) or mouse anti-FLAG M2 monoclonal antibody (for FLAG-tagged proteins) and secondary antibodies imaged by the LI-COR Odyssey system. Immunoblot input samples were normalized to contain 10 μg total protein each, which corresponds to between 2% and 4% of total protein input.

Telomerase activity and telomere length assays. The direct primer extension activity assay was performed using resin-immobilized purified holoenzyme as previously described (31). The PCR-amplified telomerase repeat amplification protocol (TRAP) activity assay was performed using total cell lysate or resin-bound samples as previously described (17). The TRAP internal control (IC) is an oligonucleotide competitor for amplification that generates a product longer than the telomerase product ladder. All activity assay samples were diluted in IP buffer. Telomere length was determined by in-gel hybridization using a 5′-end-labeled telomeric-repeat oligonucleotide as previously described (18), except for the inclusion of additional genomic DNA purification steps before and after RsaI and HinfI digestion. DNA fragment size was determined by staining an excised DNA ladder lane of the gel with ethidium bromide.

RESULTS

DHX36 association with human telomerase RNP. We probed the association of TERT and hTR with DHX36 and a panel of other helicase domain proteins, including RuvBL1 and RuvBL2, two interacting multimeric proteins previously demonstrated to play a critical role in chaperoning general H/ACA RNP biogenesis and hTR accumulation (21, 39). Comparing across the helicase domain proteins, hTR was most robustly associated with DHX36 over a range of wash stringency conditions (data not shown). Whole-cell extracts from 293T cells transiently transfected to express N-terminally zz-tagged TERT and/or N-terminally 3× FLAG-tagged (F) DHX36 were used to recover TERT on IgG-agarose and immunoblot for associated DHX36. There was not a readily detectable association between overexpressed tagged TERT and DHX36 with or without concurrent expression of additional hTR (Fig. 1B). There was also no readily detectable association if TERT was F tagged and DHX36 was TAP tagged (data not shown). On the other hand, TAP-tagged DHX36 did co-

purify hTR with or without concurrent overexpression of TERT (Fig. 1C, lanes 6 and 7 [note that mature hTR frequently migrates as a doublet due to partial folding during denaturing gel electrophoresis]). As a background control, no hTR was recovered with IgG agarose in the absence of TAP-DHX36 (Fig. 1C, lane 8). Because 293T cells have endogenous TERT, we further assessed the TERT dependence of DHX36 interaction with hTR using an RNA lacking a high-affinity TERT binding site. The hTR-U64 chimera replaces the 3′ half of hTR, including the H/ACA motif and the major TERT binding site atop the H/ACA motif 5′ stem (Fig. 1A), with the H/ACA small nucleolar RNA U64. Despite severely crippled TERT interaction (27), hTR-U64 retained robust association with DHX36 (Fig. 1C, lane 10). Together, these results support the conclusion that DHX36 associates with hTR in a manner that does not require hTR interaction with TERT.

We next investigated whether DHX36 associates with hTR in a manner that is mutually exclusive with TERT interaction and RNP catalytic activity. We compared the copurifications of hTR and telomerase catalytic activities by tagged DHX36 versus tagged dyskerin. Two subunits of dyskerin are incorporated in each biologically assembled telomerase RNP (13), so the majority hTR population of catalytically inactive RNP and the TERT-containing catalytically active holoenzyme are both enriched by the control F-tagged dyskerin. Compared to F-tagged dyskerin, F-tagged DHX36 recovered less of the overexpressed hTR (Fig. 2A, lanes 3 and 4) and proportionally less telomerase activity detected by the PCR-amplified TRAP assay (Fig. 2B, lanes 7 and 8). Nonetheless, F-tagged DHX36 did enrich both hTR and telomerase activity relative to the background detected in the absence of an F-tagged protein (Fig. 2A, compare lane 1 to lane 3; Fig. 2B, compare lane 5 to lane 7). As a specificity control, the small nuclear RNA U2 was not enriched by DHX36 or dyskerin. Overexpressing hTR increased both hTR and telomerase activity copurification with F-DHX36, but even endogenous levels of hTR and telomerase holoenzyme activity were specifically copurified (Fig. 2A, compare lanes 1, 2, and 3; Fig. 2B, compare lanes 5, 6, and 7). The RNP enriched by DHX36 appeared much lower in specific activity than the RNP enriched by TERT, suggesting that, like dyskerin, DHX36 is not preferentially associated with the catalytically active telomerase holoenzyme. These conclusions were reproducible across different extracts and tagged subunit purifications (data not shown).

Comparing the telomerase RNP purification yield from tagged dyskerin to that from tagged DHX36 is not strictly quantitative due to potential differences in tagged protein competition with endogenous untagged protein, but it seems likely that the stoichiometry of hTR association with DHX36 is less than that of its association with dyskerin. We were unable to detect specific enrichment of telomerase activity in association with endogenous DHX36 using a commercially available DHX36 antibody, potentially for technical reasons (commercial antibodies have not been reported to purify DHX36 RNP complexes). Also, because the hTR structure that recruits DHX36 would be subsequently resolved by DHX36 activity (see below), in a steady-state distribution, very little of the endogenous telomerase RNP could have associated DHX36.

RNA motif and protein domain specificity of hTR-DHX36 interaction. To address the sequence specificity of hTR-DHX36 interaction, we first considered the 5′ half of hTR that supports DHX36 interaction in the hTR-U64 chimera (Fig. 1C). Aside from the template/pseudoknot motifs required for catalytic activity in association with TERT, this region includes a putative single-stranded 5′ leader and domain-closing P1 helix that could be sites of DHX36 interaction (Fig. 3A). Notably, rodent evolution has truncated the telomerase RNA 5′ end to a starting position almost immediately 5′ of the template (5). Despite this appearance of functional dispensability, the hTR P1 helix serves an important role in template boundary definition: hTR sequence substitutions that disrupt P1 base pairing reduce the fidelity of repeat synthesis and are associated with human disease (6, 31). Synthetic RNAs harboring

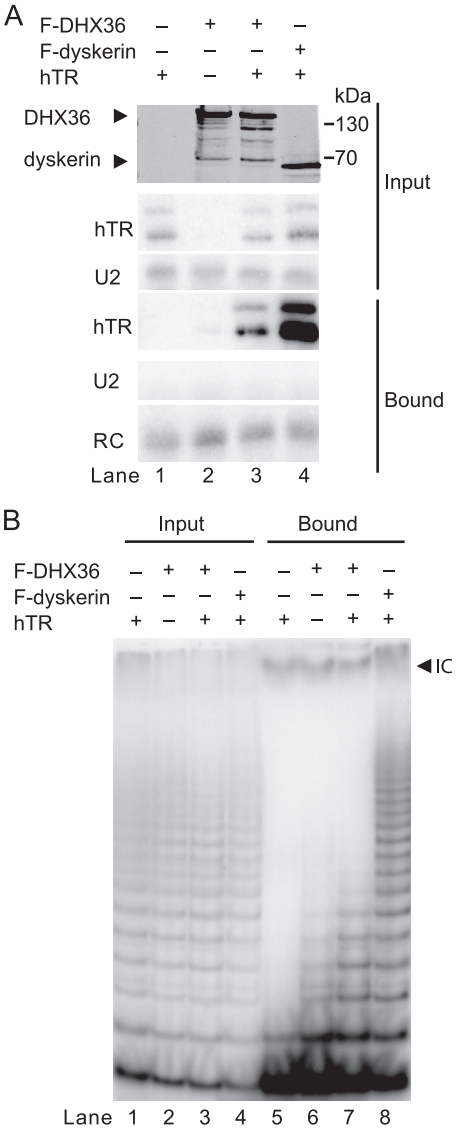

FIG. 2. DHX36 copurifies predominantly an inactive telomerase RNP population. (A) Extracts from transfected 293T cells were used to detect F-tagged protein inputs. Input and bound samples were used to detect hTR and the control U2 small nuclear RNA by blot hybridization. (B) TRAP assays of cell extract representing 0.1% of each input sample or 50% of each FLAG antibody-purified sample. IC is a TRAP assay competitive internal control.

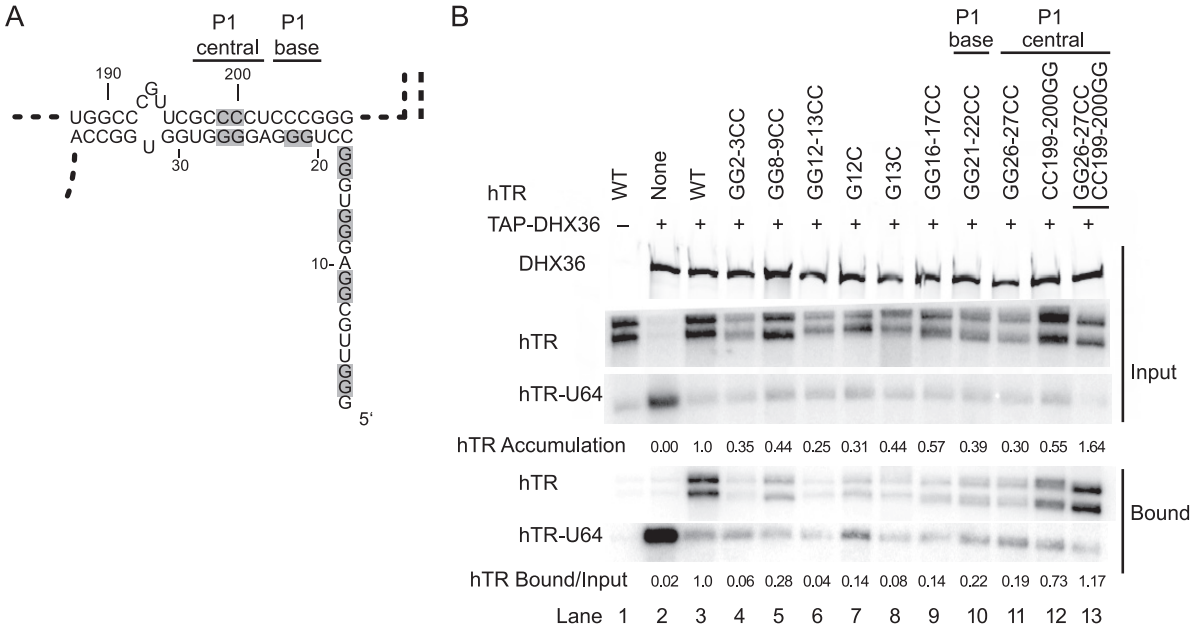

FIG. 3. DHX36 binding to hTR requires several guanosine tracts at the RNA 5′ end. (A) Schematic of hTR 5′ leader and P1 sequence. Positions of sequence substitutions are shaded. (B) Extracts from 293T cells transfected to express TAP-DHX36, hTR-U64, and wild-type (WT) or variant hTR were enriched for DHX36 using IgG-agarose and analyzed for hTR and hTR-U64 copurification by blot hybridization. Accumulation of hTR is normalized to the coexpressed hTR-U64. Bound/Input, bound hTR normalized to input hTR. All intensity values were first corrected for local background before normalization.

hTR sequences 5′ of the template can fold as a G-quadruplex (19, 26). Importantly, unlike DNA, RNA uniformly adopts the parallel-strand orientation of quadruplex *in vitro* and *in vivo* (4, 8, 30, 41). DHX36 has the ability to bind and resolve a model intermolecular G-quadruplex RNA (11). We therefore tested whether DHX36 binding requires the hTR 5′ G tracts with quadruplex-forming potential.

Tracts of two or three consecutive guanosines were substituted to replace two guanosines with two cytidines (pairs of substituted guanosines are shaded in Fig. 3A). We coexpressed wild-type or variant hTR with hTR-U64 as an internal transfection control, recovered DHX36-associated RNAs by immunopurification, and quantified the input and bound hTR and hTR-U64. Most of the hTR G tract substitutions reduced mature hTR accumulation relative to coexpressed hTR-U64 (Fig. 3B [hTR accumulation, hTR level normalized to hTR-U64]). We then determined the relative percentage of input hTR associated with DHX36 (Fig. 3B [hTR bound/input]). Substitutions GG(2,3)CC, GG(12,13)CC, and GG(16,17)CC in the 3-G tracts of the 5′ leader drastically reduced hTR-DHX36 interaction, as did the G12C and G13C single-nucleotide substitutions of the central 3-G tract (Fig. 3B, lanes 3, 4, and 6 to 9). For these hTR variants, the amount of bound hTR approached the low level of the background from copurification of endogenous wild-type hTR (Fig. 3B, lane 2). The substitution GG(8,9)CC in the only 2-G tract of the 5′ leader reduced DHX36 interaction to a lesser extent (Fig. 3B, lane 5). Within the first few base pairs of P1, the GG(21,22)CC substitution strongly inhibited DHX36 interaction (Fig. 3B, lane 10). A more central P1 substitution, GG(26,27)CC, reduced DHX36 interaction more than did the CC(199,200)GG substitution of the complementary strand, but the compensatory combination of substitutions rescued DHX36 interaction with hTR (Fig. 3B, lanes 11 to 13). These results implicate several guanosine tracts near the hTR 5′ end as contributors to DHX36 interaction.

Potentially, any combination of four-G tracts at a nascent hTR 5′ end could fold as an intramolecular quadruplex to form a DHX36 binding site. The results above suggest that folding favors inclusion of the 5′ leader 3-G tracts GGG(1–3), GGG(11–13), and GGG(15–17). In alternative folds, the fourth strand could be contributed by GG(8,9), GGG(21–23), or GGGG(25–28). Formation of P1 would disfavor incorporation of the central P1 GGGG(25–28) tract more than the GGG(21–23) tract toward the base of the stem. Indeed, results from hTR chemical modification and enzymatic digestion after folding *in vitro* or *in vivo* support complete base pairing of central P1 GGGG(25–28) but a more dynamic or mixed population of structures involving GGG(21–23) and/or the partner cytidines at the base of P1 (1). Curiously, all of the multiple-guanosine tracts in the hTR 5′ leader are partially protected from modification compared to guanosines around the template, and the GGG(11–13) and GGG(15–17) tracts are sites of pauses to reverse transcription. The only hTR 5′-end guanosine not protected from modification after folding *in vitro* or *in vivo* is the singlet G6 (1), which substituted in G6C hTR had no impact on DHX36 interaction (data not shown). Together, the previous findings and our results above suggest that at least some hTR RNPs have a folded guanosine tract structure(s) recognized by DHX36.

To investigate the DHX36 domain requirements for hTR interaction, we compared hTR copurifications with full-length and truncated DHX36 proteins (Fig. 4A), with or without the E335A active-site substitution that abrogates helicase catalytic activity (22). The unique N-terminal domain of DHX36 alone

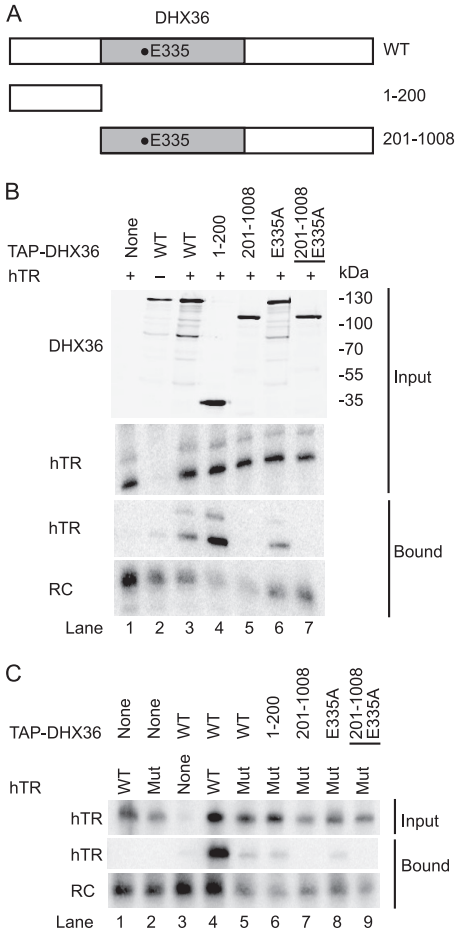

FIG. 4. The N-terminal domain of DHX36 is necessary and sufficient for sequence-specific association with hTR. (A) Schematic of DHX36 protein domain expression. The helicase motif region is shaded, and the position of the E335 substitution that abrogates catalytic activity is indicated. (B and C) Extracts from transfected 293T cells expressing recombinant wild-type (WT) or G12C (Mut) hTR and full-length, truncated, and/or substituted TAP-DHX36 were enriched using IgG-agarose and analyzed for hTR copurification by blot hybridization.

(amino acids 1 to 200) copurified hTR as well as or better than the full-length protein, whereas the helicase domain (amino acids 201 to 1008) did not support any detectable hTR interaction (Fig. 4B, lanes 1 to 5). The catalytically dead full-length protein retained hTR interaction comparable to that of wild-type DHX36, and catalytically dead helicase domain did not gain hTR interaction (Fig. 4B, lanes 6 and 7). The DHX36 N-terminal domain alone showed the same specificity of hTR interaction as full-length DHX36, with binding abrogated by the G12C hTR substitution (Fig. 4C, Mut). In cells expressing G12C hTR, tagged DHX36 enriched only the low level of endogenous hTR that also copurified with DHX36 in the absence of any recombinant hTR (Fig. 4C, lanes 3 and 5). Likewise, from input extracts containing abundant G12C hTR, the DHX36 N-terminal domain and the catalytically dead full-length protein copurified only a low level of hTR likely to be entirely of endogenous wild-type sequence (Fig. 4C, lanes 6 and 8). In comparison, the DHX36 helicase domain copurified neither recombinant G12C hTR nor endogenous wild-type hTR (Fig. 4C, lanes 7 and 9).

These results extend previous studies of DHX36 RNA binding specificity *in vitro*, which revealed that the DHX36 N-terminal domain is sufficient for binding to the intermolecular G-quadruplex model substrate that can be resolved by the helicase domain in full-length DHX36 (11, 25). Different from previous findings, we did not detect increased RNA association with full-length DHX36 compared to that of the DHX36 N-terminal domain alone. The previously inferred contribution of the helicase domain to G-quadruplex binding may have been a consequence of a difference in folding of the recombinant full-length protein expressed in insect cells versus the recombinant N-terminal domain expressed in *Escherichia coli*, or it could reflect helicase domain binding to the long single-stranded regions of the model quadruplex generated by intermolecular annealing of an $A_{15}G_5A_{15}$ oligonucleotide.

Biological function of the hTR guanosine tract motif. To address the functional role of the hTR G tracts recognized by DHX36, we first assessed whether disruption of these sequences affected hTR-TERT interaction or the catalytic activity of holoenzyme assembled *in vivo*. We coexpressed each of a panel of hTR sequence variants with tagged TERT in 293T cells, recovered the F-tagged TERT, and assayed for coenrichment of hTR. As predicted from the known hTR determinants of TERT binding (27), none of the hTR 5′-end sequence substitutions prevented hTR-TERT interaction (Fig. 5, top). Furthermore, in agreement with established requirements for activity reconstitution using TERT and minimized hTR domains *in vitro* (7, 27), none of the hTR 5′-end sequence substitutions notably inhibited holoenzyme activity or repeat addition processivity (Fig. 5, bottom). As expected based on the role of P1 in template 5′ boundary definition (6, 31), unpairing central P1 increased the synthesis of products 1 nucleotide longer than the normal ladder (Fig. 5, lanes 6 and 7 [longer products are marked with a filled arrowhead]). Compensatory stem substitutions that repaired central P1 suppressed this defect (Fig. 5, lane 8). Template boundary bypass was less severe in the hTR GG(21,22)CC holoenzyme disrupted for pairing of the P1 base (Fig. 5, lane 5), consistent with the relatively modest template 5′ boundary bypass imposed by the disease-linked hTR C204G variant compared to the 5′ boundary bypass imposed by a P1 disruption closer to the template (31).

We next investigated the impact of G tract disruptions on hTR function in telomere maintenance. Some but not all cell lines will show an increase in the steady-state accumulation of mature hTR when hTR precursor is overexpressed from an integrated retroviral expression construct. Fibroblasts from X-linked dyskeratosis congenita patients that ectopically express TERT maintain exceptionally short telomeres due to the limiting level of endogenous hTR, and so additional hTR expression induces rapid telomere elongation to a stably maintained longer telomere length set point (40). This provides a sensitized system for detecting even partial inhibition of hTR function in telomere elongation (14). We stably transduced the 1774TERT X-linked dyskeratosis congenita fibroblast cell line with the negative-control empty vector, positive-control wild-type hTR expression vector, and expression vectors encoding hTR variants that compromise the DHX36 interaction indic-

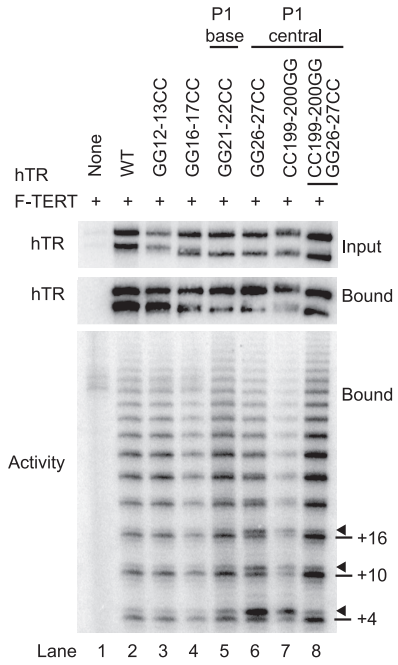

FIG. 5. Variants of hTR with a disrupted G tract motif and loss of DHX36 association retain TERT association and support telomerase holoenzyme catalytic activity. Extracts from transfected 293T cells were enriched for F-TERT and assayed for copurification of hTR by blot hybridization. Purified telomerase holoenzymes were then assayed for telomerase activity by direct extension of the telomeric-repeat primer $(T_2AG_3)_3$, with products resolved by denaturing gel electrophoresis. The predominant products from complete repeat synthesis are indicated by the number of nucleotides added to the primer (+4, +10, +16), while products 1 nucleotide longer due to template boundary bypass synthesis are indicated with a filled arrowhead.

ative of G-quadruplex formation [GG(12,13)CC, G12C, GG(16,17)CC, and GG(21,22)CC]. Following selection for retroviral integration, polyclonal cell populations were harvested at increasing population doublings to assay hTR accumulation, telomerase activity in cell extract, and telomere length. As observed previously (14, 40), each cell line rapidly reached a new telomere length set point (data not shown).

Each of the hTR G tract variants accumulated at a reduced steady-state level compared to wild-type hTR accumulation although still substantially above the endogenous hTR level in cells transduced with the empty vector (Fig. 6A). Consistent with the levels of hTR accumulation, cell lines expressing hTR G tract variants had levels of telomerase activity in cell extract that were greater than that of the empty vector control, as detected by TRAP assay (Fig. 6B). The cell lines expressing hTR G tract variants stably maintained telomeric restriction fragment lengths (Fig. 6C, lanes 3 to 6) that were intermediate between those of the cells expressing empty vector (lane 2) and the cells expressing wild-type hTR (lane 1). We repeated this test of hTR function for the G12C hTR variant in HT-1080 human fibrosarcoma cells. Integration of the wild-type hTR expression construct produced a much more modest increase in steady-state hTR accumulation and telomerase activation than was obtained in 1774TERT cells, likely due to the higher endogenous level of hTR (Fig. 7A and B). As in the primary fibroblast system, compared to the empty vector control, HT-1080 cells stably expressing G12C hTR elongated telomeres, but the extent of telomere elongation was reduced in comparison to elongation in cells expressing additional wild-type hTR (Fig. 7C [duplicate lanes are independent loading]). In summary, in both cell types, hTR G tract variants supported a reduced level of telomere elongation due in part or whole to compromised hTR accumulation.

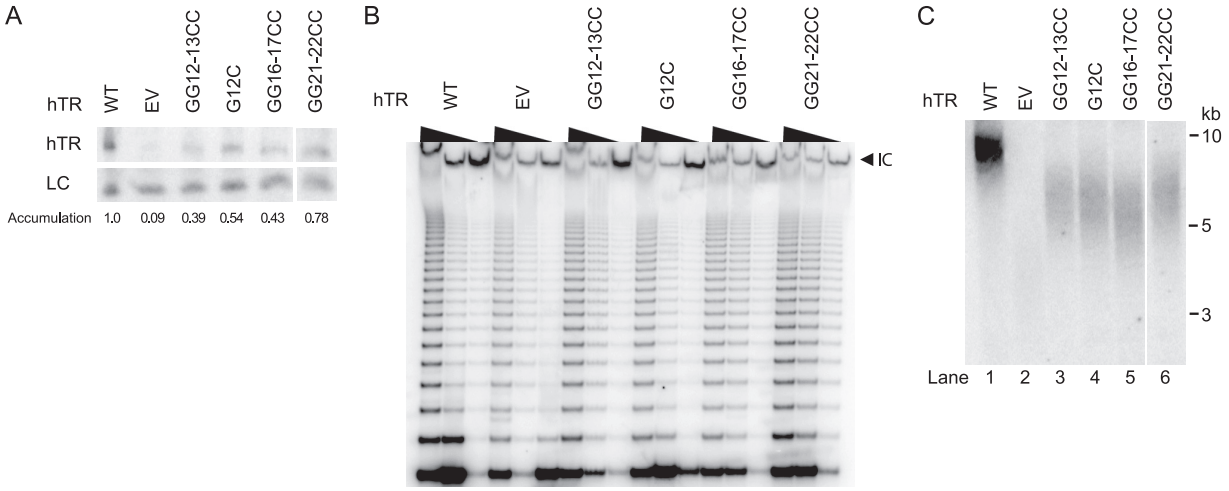

FIG. 6. Substitutions in the 5′ guanosine tracts reduce hTR steady-state accumulation and telomere maintenance in 1774TERT fibroblast cell lines. Note that adjacent panels were cropped from the same blot exposure. (A) Total RNA extracted from 1774TERT cell lines expressing the indicated form of recombinant hTR (WT, wild type; EV, empty vector) was used to detect hTR by blot hybridization. A cross-reacting RNA provides the loading control (LC) used to normalize relative hTR accumulation. (B) TRAP assay detection of telomerase activity from cell extracts using 2 μg total protein and sequential 1:3 dilutions. (C) In-gel hybridization of telomeric restriction fragments was used to compare telomere lengths. Note that adjacent panels were cropped from the same blot exposure. Analysis of multiple time points of culture growth (not shown) confirmed that these telomere lengths are stably maintained in telomere length homeostasis.

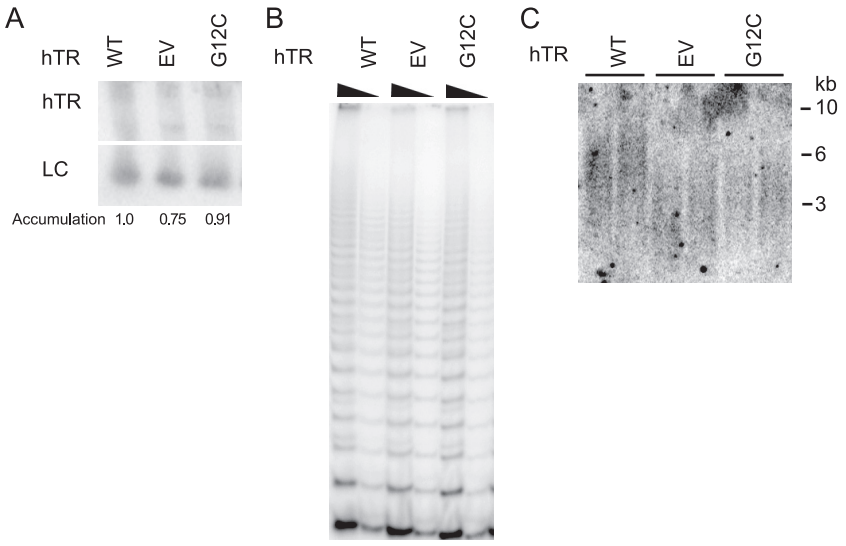

FIG. 7. Substitution G12C reduces hTR steady-state accumulation and telomere maintenance in HT-1080 cells. (A) Total RNA extracted from HT-1080 cell lines expressing the indicated form of recombinant hTR was used to detect hTR and the loading control by blot hybridization. (B) TRAP assay detection of telomerase activity from whole-cell extracts using 1 μg total protein and a 1:10 dilution. (C) In-gel hybridization of telomeric restriction fragments was used to compare telomere lengths. Duplicate lanes are independent loadings of the same genomic DNA digest. Analysis of multiple time points of culture growth (not shown) confirmed that these telomere lengths are stably maintained in telomere length homeostasis.

DISCUSSION

Proteins required for hTR biogenesis and accumulation can limit telomerase-mediated telomere maintenance, as evident in inherited diseases of human telomerase deficiency (32). Here, we uncover a previously unsuspected recognition of hTR by the DEXH box RNA helicase DHX36. Consistent with previous studies of DHX36 activity as a G-quadruplex binding factor (11, 37), we found that DHX36-hTR interaction requires multiple G tracts at the hTR 5′ end. Also consistent with a concurrent study (25), our results suggest that the N-terminal domain of DHX36 is necessary and sufficient for association with G tract RNA. The hTR binding site for DHX36 contains closely spaced G tracts that would be favored by dilute concentration *in vivo* to fold as an intramolecular quadruplex, in contrast to the $A_{15}G_5A_{15}$ model substrate forced to fold as an intermolecular quadruplex *in vitro*. Nonetheless, the highly preferred parallel-strand orientation of G-quadruplex RNA would give the model RNA oligonucleotide and endogenously folded hTR a similar structure of stacked guanosines for recognition by DHX36 (8).

Using hTR variants that retain TERT interaction and holoenzyme catalytic activity, we examined the significance of the hTR G tracts in cellular context. G tract substitutions, including the single-nucleotide change G12C, reduced hTR function in telomere elongation in part or whole by reducing the steady-state level of mature hTR. The decrease in RNP accumulation could be due to loss of DHX36 interaction, but we suspect instead that DHX36 binds hTR in the process of resolving a G-quadruplex structure that itself is the determinant of RNP accumulation. Because the majority of hTR RNP is not bound by DHX36 at steady state yet disruption of the DHX36 interaction site on hTR severely reduces mature hTR accumulation, we propose that a large fraction of hTR precursor folds to contain a quadruplex that acts transiently in an early stage of RNP biogenesis to protect the RNA from degradation. This quadruplex structure would subsequently be recognized and likely unfolded by DHX36, resulting in DHX36 release. The unstructured hTR 5′ region could potentially refold as quadruplex, but this may often be competed by formation of a complete P1. A quadruplex involving only the 5′ leader from P1 would fold with less than optimal stability due to the necessary inclusion of a 2-G tract. Also, the mature hTR trimethylated 5′ cap structure could diminish the stability of quadruplexes formed with the 5′ guanosine. DHX36 recognition and resolution of a structure formed by the majority of nascent hTR transcripts would account for the highly stabilizing biological role of the hTR guanosine tracts and yet the low steady-state level of DHX36 association with active telomerase RNP. Vertebrate telomerase RNAs that lack a guanosine-rich 5′ leader could compensate for this loss by increased precursor expression or other stabilizing influences, such as more rapid 5′ cap modification.

DHX36 has a primarily nucleoplasmic but partially cytoplasmic distribution and has multiple ascribed functions in the regulation of mRNA expression and turnover (22, 35). The pleiotropic impact of DHX36 depletion on mRNA levels complicates any analysis of a direct influence of DHX36 on telomere biology, particularly given that only a minor fraction of the total cellular DHX36 would be required to saturate interaction with the scarce telomerase RNP. Because DHX36 recognition of hTR is mediated by the N-terminal protein domain, the helicase domain would be poised for association with nearby potential substrates. In addition to acting as a resolvase for structured regions of hTR, more speculatively, the helicase domain of hTR-bound DHX36 could act on telomeric DNA or telomeric RNA quadruplexes. DHX36 resolution of telomeric

RNA or telomeric DNA quadruplexes could be expected to activate telomerase for telomere elongation (4, 15), with the prediction that disruption of the hTR binding site for DHX36 would result in telomere shortening.

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