The Nucleolar Localization Domain of the Catalytic Subunit of Human Telomerase*

Katherine T. Etheridge‡, Soma S. R. Banik‡, Blaine N. Armbruster‡, Yusheng Zhu§, Rebecca M. Terns§, Michael P. Terns§, and Christopher M. Counter‡‡

From the ‡Departments of Pharmacology and Cancer Biology and Radiation Oncology, Duke University Medical Center, Durham, North Carolina 27710 and the §Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602

Received for publication, February 26, 2002

Published, JBC Papers in Press, April 15, 2002, DOI 10.1074/jbc.M201227200

Telomerase is the enzyme essential to complete the replication of the terminal DNA of most eukaryotic chromosomes. In humans, this enzyme is composed of the telomerase reverse transcriptase (hTERT) and telomerase RNA (hTR) subunits. hTR has been found in the nucleus, a site of assembly of ribosomes as well as other ribonucleoproteins (RNPs). We therefore tested whether the hTERT component is also found in the nucleus, where it could complex with the hTR RNA to form a functional enzyme. We report here that hTERT does indeed localize to the nucleus, and we mapped the domain responsible for this localization to the hTR-binding region of the protein by deletion analysis. Substitution mutations in two of the three conserved hTR-binding domains in this nucleolar localization domain (NoLD) abolished nucleolar localization. However, another mutation that impeded hTR binding did not alter this subcellular localization. Additionally, wild type hTERT was detected in the nucleus of cells that failed to express hTR. Taken together, we propose that the nucleolar localization of hTERT involves more than just the association with the hTR subunit. Furthermore, the coincidental targeting of both the hTR and hTERT subunits to the nucleus supports the premise that the assembly of telomerase occurs in the nucleus.

Telomerase is a reverse transcriptase ribonucleoprotein (RNP) complex composed of a reverse transcriptase catalytic protein subunit (TERT) that copies a template region of an RNA subunit (TR) onto telomeres as DNA (1). In humans, this enzyme is of great medical importance because of its pivotal role in unlimited cellular proliferation, a hallmark of cancer cells (2). The union of the RNA and protein subunits to form an RNP is essential for telomerase activity (3).

Based on a comparison of the amino acid sequence of TERT from organisms of many different kingdoms and on mutational analysis, it has been possible to identify a number of discrete domains in TERT. The central region of the catalytic subunit contains seven motifs found in reverse transcriptases, which define the catalytic core (4–17). The C terminus of TERT is highly divergent, both at the sequence and the functional level (18, 19). The N-terminal region is more conserved, containing domain I and the TAT (dissociates activities of telomerase) domain (18, 20) followed by domains II and III (18, 20, 21) and the T motif (1, 5, 6), which are essential for telomere elongation. Substitution mutations in domains II and III or the T motif decrease hTERT binding to the telomerase RNA in yeast (18), ciliate (22, 23), or human cells (20, 24) and correspondingly result in a dysfunctional enzyme. Deletion analysis has also defined the region extending from amino acids 326 to 613, which harbors all three of the aforementioned domains, as the minimum region required for hTR binding (22), although mutations in domain I can also have some effect on hTR binding (24).

The site of assembly of the telomerase RNP has not been determined; however, there is growing evidence supporting a connection to the nucleus in vertebrate systems. The nucleus is well known as the site of ribosome assembly and has been speculated to be a site for the assembly of other RNPs (25–27). The hTR RNA component of human telomerase contains a sequence/structure motif characteristic of Box H/ACA small nucleolar RNAs, which guide RNA processing and modification within the nucleus (28–30). Moreover, hTR co-immunoprecipitates the small nucleolar RNA-binding proteins dyskerin (31), GAR1 (32), NHP2, and NOP10 (33, 34), indicating that hTR can exist in a complex with these nucleolar proteins. Lastly, hTR has been found to localize to the nucleus by virtue of its Box H/ACA motif (28–30). However, the same may not be true in lower eukaryotes. For example, yeast telomerase RNA lacks a Box H/ACA motif (28) but instead shares features of splicingosmal small nucleolar RNAs (35, 36). Therefore, unlike other functions of telomerase, understanding the biogenesis of this enzyme in humans may be achieved only by studying this process in higher eukaryotes. This is of particular importance because improper telomerase RNP accumulation has been linked to the human disease dyskeratosis congenita (31, 37).

Given that hTR is found in the nucleus, a site for the assembly of ribosomes and possibly other RNPs (25–27), we tested whether the hTERT catalytic subunit is also found in this subnuclear compartment by monitoring the subcellular localization of hTERT when fused to the yellow fluorescent protein (YFP) in human cells. We found that hTERT localized to the nucleus, and we mapped the corresponding nucleolar localization domain to the region encompassing the N-terminal domains II and III and the T motif. Although point or substitution mutations in any of these three domains decreased hTR binding in vitro, not all of these mutations disrupted hTERT nucleolar localization, suggesting that localization can occur independent of hTR binding. Indeed, hTERT was found in the...
nuclei of human cells that do not express hTR. These results demonstrate that hTERT contains a discrete nucleolar localization domain (NoLD) that targets the molecule to the nucleolus and that this subcellular distribution can be mediated through interactions with factors other than hTR.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human osteosarcoma cell line U2OS, the human cervical adenocarcinoma cancer cell line HeLa, and the SV40 transformed human fibroblast cell lines WI38 VA13/2RA (American Type Culture Collection) and LM217 (38) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

**Plasmids**—pYFP-hTERT, hTERT-II<sub>386</sub>, or TERT-III<sub>512</sub>, were made by subcloning the EcoRI/SalI FLAG-hTERT cDNA either in the wild type format or with a six-aminoc acid (NAAIRS) substitution of the sequence pS-YGWMP in domain II or pS5-MSWRGC in domain III, respectively (20), into the same sites of plasmid pEYFP-C1 (CLONTECH). FLAG-tagged NoLD was made by PCR-amplifying hTERT cDNA with the primers 5'-GGGATCCACCATGGACTACAAAGACGATGACGAC-3' and 5'-CCTCGAGTCGACGACTAGTGGCG-3'. The same six aminoc acids described above beginning at positions 386 and/or 512 were substituted with the sequence NAARIS, and/or amino acid Phe<sup>561</sup> in the T motif. The same sites of pCI-neo (Promega) for the above described plasmids encoding the appropriate YFP fusion protein using either the FuGENE 6 (Roche Molecular Biochemicals) or the same sites of LipofectAMINE 2000 (Invitrogen) reagents according to the manufacturer’s protocols. After 48 h, live U2OS or LM217 cells were observed under PBS at ×40 magnification on a Zeiss Axioskop fluorescence microscope. VA13 and HeLa cells were fixed in 4% formaldehyde in PBS with 0.1% Triton X-100, and cloned into the same sites in pEYFP-C1.

hTR-hTERT Co-immunoprecipitation—As described previously (20), hTERT was transcribed and <sup>32</sup>P-labeled with the T7-coupled Maxiscript (Ambion) using 1 µg of linearized pBluescriptSK-hTR plasmid (20), after which unincorporated nucleotides were removed using a G-25 minispin column (Amersham Biosciences). FLAG-tagged NoLD, either wild type or with the described mutations, was expressed from the T7 promoter of pCI-neo and 35S-labeled in the presence of 100 ng/ml glycogen in PBS supplemented with 1.5 mM dithiothreitol, 0.5% SDS buffer, and resolved by SDS-PAGE. TNT reactions were diluted in supplemented PBS with nonspecific blockers and 200 units of RNasin (Promega) and immunoprecipitated at room temperature for 1 h with M2-agarose. The agarose beads were then washed three times with prechilled supplemented PBS, heated in SDS buffer, and resolved by SDS-PAGE.

Detection of hTR RNA—Water or 100 ng of total RNA isolated with the RNazol reagent according to the manufacturer’s instructions (Tel-Test) from WI38 VA13/2RA or telomerase-positive PC3 (39) cells was reverse transcribed and PCR amplified to detect either total hTR or porphobilinogen deaminase (PBGD) mRNA using the LightCycler TeloTAGGG hTR quantification kit and the LightCycler system in accordance with the manufacturer’s instructions (Roche Molecular Biochemicals).

**Visualization of YFP-tagged Proteins and Fibrillarin**—Localization of YFP fusion proteins was visualized in U2OS, LM217, HeLa, or VA13 cells, after which a minimum of 100 cells with the nuclear YFP protein were scored for nucleolus staining and expressed as a percent based on the total number of cells observed.

**FIG. 1. hTERT is found in the nucleolus.** A, an example of a U2OS cells transiently expressing YFP-hTERT and stained with an anti-fibrillarin antibody to detect the nucleolar protein fibrillarin (left) or viewed as a fluorescent image to detect YFP-hTERT (middle) or a merge of both images. B, an example of a U2OS, LM217, or HeLa cell transiently expressing YFP-hTERT is shown as a differential interference or phase contrast image (left) to visualize nucleoli (arrows) or as a fluorescent image (right) to visualize the YFP-tagged protein.

| YFP fusion     | Cell type | Nucleolus<sup>a</sup> | %   |
|----------------|-----------|------------------------|-----|
| hTERT          | U2OS      | 82                     | 82  |
|                | LM217     | 96                     | 96  |
|                | HeLa      | 93                     | 93  |
|                | VA13      | 80                     | 80  |
| hTERT-II<sub>386</sub> | U2OS      | 57                     | 57  |
| hTERT-III<sub>512</sub> | U2OS      | 73                     | 73  |

<sup>a</sup> Only cells with nuclear YFP were scored (full-length hTERT was found in the nucleus of ~80% of transfected cells). Wild type full-length hTERT was found in the nucleolus and the nucleolus+nucleoplasm; hence, only cells with such staining were scored as positive for being in the nucleolus.
meabilized with 0.5% Nonidet P-40 in 1× PBS and incubated with the human polyclonal anti-fibrillarin antibody (40) at 1:500 dilution. The primary antibody was detected with the rhodamine (TRITC)-conjugated donkey anti-human IgG antibody (Jackson ImmunoResearch Laboratories) and visualized as above.

RESULTS AND DISCUSSION

hTERT Is Found in the Nucleolus—To address whether hTERT is found in the nucleolar compartment of the cell, YFP was fused in-frame with the N terminus of full-length hTERT. We chose to monitor hTERT localization by detecting YFP because the fusion of large polypeptides at the N terminus has no measurable effect on telomerase activity (Ref. 41 and data not shown), YFP-tagged proteins can be visualized in live cells (42), and there is an absence of antibodies readily capable of detecting endogenous hTERT at the subcellular level. The YFP-hTERT construct was transiently expressed in the human osteosarcoma cell line U2OS because these cells lack telomerase activity (43), which eliminates possible interference by multimerization with the endogenous hTERT protein (20, 44–46). The subcellular distribution of YFP-hTERT was then assayed by fluorescence light microscopy. We found that YFP-hTERT is located predominantly in the nucleolus or both the nucleolus and nucleoplasm as assessed by co-localization with

![Deletion mapping identifies the nucleolar localization domain of TERT.](image)

**FIG. 2.** Deletion mapping identifies the nucleolar localization domain of TERT. **A,** a scale diagram representing the known regions of hTERT (refer to text for description) is shown above a line diagram depicting the size and position of hTERT fragments that were fused to YFP. The presence (+) or absence (−) of nucleolar localization of these fragments when expressed in U2OS cells is shown at the right. The putative nucleolar localization domain (NoLD) is highlighted in gray. **a,** amino acids. **B,** an example of a U2OS cell transiently expressing the described hTERT fragments fused to YFP is shown as a phase contrast image (top) to visualize nucleoli or as a fluorescent image (bottom) to visualize the YFP-tagged proteins.

![Table II](image)

**TABLE II**

Subnuclear localization of YFP-tagged NoLD proteins

| YFP fusion | Cell type | Nucleolus |
|------------|-----------|-----------|
| NoLD       | U2OS      | 100       |
| NoLD       | LM217     | 99        |
| NoLD       | HeLa      | 95        |
| NoLD       | VA13      | 97        |
| NoLD-II    | U2OS      | 29        |
| NoLD-II    | LM217     | 35        |
| NoLD-III   | U2OS      | 70        |
| NoLD-III   | LM217     | 74        |
| NoLD-I     | U2OS      | 25        |
| NoLD-I     | LM217     | 34        |
| NoLD-I     | HeLa      | 5         |
| NoLD-I     | VA13      | 6         |
| NoLD-II +  | U2OS      | 26        |
| NoLD-II +  | U2OS      | 20        |
| NoLD-II +  | LM217     |            |
| NoLD-II +  | HeLa      |            |
| NoLD-II +  | VA13      |            |

*See Fig. 3A for a description of the fusion constructs.

*Wild type NoLD was found primarily in the nucleolus; hence, only cells with predominant nucleolar staining were scored as positive for being in the nucleolus.
the nucleolar protein fibrillarin detected by indirect immunofluorescence (40) or by co-localization with nucleoli as identified using differential interference or phase contrast optics (Fig. 1, A and B, and Table I).

To rule out the possibility that this observation was unique to these cells, we introduced YFP-hTERT into another telomerase negative human cell line, LM217 (38). Consistent with our observations using U2OS cells, YFP-hTERT was found predominantly in the nucleolus. YFP-hTERT was even detected in the nucleolus of the telomerase-positive line HeLa (Fig. 1B and Table I). We therefore conclude that hTERT is found in the nucleolus and that this event is independent of cell type.

The Nucleolar Localization Domain of hTERT Encompasses Domains II and III and the T Motif—To map the region of hTERT required for nucleolar localization, we fused YFP to a series of fragments that represent key regions of hTERT and collectively span the entire length of the protein (Fig. 2A). Each of these constructs was introduced into U2OS cells, and >100 transfected cells were scored for subcellular localization. Fragment 1–183, which encompasses domain I and the DAT domain, did not localize to the nucleolus. Similarly, fragment 523–924, encoding the T motif and all the reverse transcriptase domains, or fragment 867–1132, encompassing the entire C terminus, also failed to localize to the nucleolus (Fig. 2B). Since all of these fragments were detected in the nucleus, we discount the possibility that the peptides were excluded from the nucleolus due to a failure to enter the nucleus. We therefore surmise that neither the DAT domain, the catalytic core, nor the C terminus of hTERT contains a nucleolar localization domain.

However, fragment 326–620, which encodes the hTR-binding domains of hTERT (domains II and III and the T motif), was always found in the nucleolus (Fig. 2B and Table II), indicating that this fragment contains sequences sufficient for nucleolar targeting. We found that the overlapping fragment 170–546,
which contains domains II and III, or the fragment encompassing the catalytic region (523–924), which also contains part of domain III and the T motif, was not localized to the nucleolus. We therefore reasoned that at least two elements are required for nucleolar targeting of the 326–620 region encompassed by domain II to the T motif. We term this portion of hTERT the nucleolar localization domain (NoLD). We next confirmed that the NoLD was localized to the nucleolus in other cell types. Specifically, we expressed the YFP-NoLD polypeptide in LM217 and HeLa cells and again found the YFP-tagged protein in the nucleolus of 99–100% of the cells (Table II). Thus, the NoLD encodes a potent nucleolar localization sequence that functions regardless of cell type.

Mutations in Domain II and the T Motif Affect the Nucleolar Targeting of hTERT—The NoLD encompasses conserved sequences denoted as domains II, III, and the T motif. These domains also map to regions determined by mutational analysis to be essential for telomerase activity in humans, yeast, or ciliates (18, 20–23, 45, 47). We have previously shown that a six-amino acid substitution (with the sequence NAIRS) beginning at position 386 in domain II or 512 in domain III of hTERT reduces the association of this protein with hTR (20). Similarly, an alanine substitution of the highly conserved phenylalanine residue in the T motif is known to decrease telomerase RNA binding in ciliates (23). Collectively, these data argue that these three domains are responsible for most telomerase RNA binding. Because nucleolar localization was detected with a fragment encompassing the sequences encoding the hTR RNA-binding activities of hTERT (but not with fragments outside this region) and hTR is known to localize to the nucleolus (28–30), we queried whether the RNA-binding activity of hTERT was essential for the observed nucleolar localization of hTERT. We introduced the aforementioned mutations in domains II, III, and the T motif one at a time or in combination into the NoLD fragment of hTERT (Fig. 3A). We then confirmed that the NoLD fragment harboring mutations in domains II and III and the T motif abolished hTR binding in vitro. Specifically, [35S]labeled FLAG-tagged NoLD fragments, either in the wild type format or with the 386, 512, or 561 mutations, were incubated with [32P]-labeled hTR and immunoprecipitated with an anti-FLAG antibody. The FLAG-tagged NoLD polypeptide, but not an irrelevant FLAG-tagged protein (HDAC1), co-immunoprecipitated hTR. However, mutations in any of the three hTR-binding domains abolished this association with no further loss observed, even when all three mutations were introduced into the same polypeptide (Fig. 3B). Thus, we confirm that mutations 386, 512, and 561 abolish detectable hTR association with the NoLD in vitro.

Having confirmed that the mutations abolish detectable hTR binding, we next monitored the subcellular localization of the YFP-tagged proteins in U2OS cells by fluorescence microscopy. We have found that a substitution mutation in domain II (mutant 386), which eliminated hTR binding and is known to abolish the catalytic function of hTERT (Fig. 3B and Ref. 20), greatly reduced the nucleolar localization of the NoLD fragment (Fig. 4 and Table II). We confirmed that the NoLD fragment harboring mutations in domain II, III, and the T motif one at a time or in combination greatly reduced the nucleolar localization of the NoLD fragment (Fig. 4 and Table II). We found that the mutations 386, 512, and 561 abolished this observed nucleolar localization, and these effects were independent of cell type.

Mutations in More than One hTR-binding Domain Do Not Further Disrupt Nucleolar Localization—We next addressed whether mutations in more than one hTR-binding region fur-
ther decreased nucleolar localization. YFP was fused to a series of NoLD fragments containing different combinations of domains II and III and T motif mutations (Fig. 3A). The resultant constructs were then expressed in U2OS cells and assayed for nucleolar localization. We found that mutating either two or all three hTR-binding domains did not have an additive effect. A mutation in domain II disrupted the nucleolar localization of the NoLD fragment to approximately the same degree whether domain III or the T motif or both were also mutated (Table II). The same was true with the T motif; additional mutations did not further diminish nucleolar localization (Table II). These results are consistent with a model whereby mutations in domains II or the T motif are alone capable of disrupting the accumulation of hTERT in the nucleolus.

**The NoLD Is Required for Nucleolar Localization of Full-length hTERT**—Mutation in domain II greatly reduced the nucleolar accumulation of the NoLD fragment when expressed in human cells. To directly test whether this domain was essential to target full-length hTERT to the nucleolus, we created a fusion of the YFP with hTERT containing the aforementioned NAAIRS substitution mutation in domain II. The fusion protein was transiently expressed in U2OS cells and assayed for subcellular localization. Although the full-length hTERT molecule was found in the nucleolus, the introduction of a NAAIRS substitution mutation at position 386 in domain II reduced this localization by almost one-half (Fig. 5 and Table I). Thus, we conclude that the NoLD identified by deletion analysis mediates the localization of target full-length hTERT to the nucleolus.
The hTERT Nucleolar Localization Domain

hTERT Nucleolar Localization Is Independent of hTERT Binding—We have shown that the nucleolar localization domain of hTERT maps to the region of the protein linked to hTERT binding (Fig. 2) and that mutations in two known hTERT-binding domains, domain II and the T motif, crippled the ability of the protein to accumulate in this compartment of the nucleus. Such observations are consistent with the hTERT recruiting hTERT to the nucleolus via the Box H/ACA motif. However, one mutation that abolished all measurable association of hTERT with hTR (Fig. 3B) had very little effect on the accumulation of the NoLD fragment (Fig. 4) or full-length hTERT (Table I) in the nucleolus. We interpret these data in one of two ways. Interaction with hTR may not be required for targeting hTERT to the nucleolus. Alternatively, hTERT binding may be essential for hTERT localization, but the mutation in domain III does not disrupt hTERT binding to the same extent in vivo as observed in vitro. To differentiate between these two models, YFP-tagged full-length hTERT and the NoLD fragment were introduced into WI38 VA13/2RA human cells. These cells reportedly lack both hTERT and hTR transcripts (48, 49) and require the ectopic expression of these two subunits to restore telomerase activity (49). Indeed, we confirm by the exquisitely sensitive real-time quantitative reverse transcription-PCR that the hTERT transcript is not present in these cells despite being readily detected in telomerase-positive cells (Fig. 6A). If hTERT binding is indispensable for the nucleolar localization of hTERT, then the hTERT or NoLD proteins should not be detected in the nucleolus when expressed in these cells. However, we find that ectopically expressed YFP-hTERT as well as the YFP-NoLD itself was found almost exclusively in the nucleolus, supporting the premise that hTERT can be targeted to this subcellular structure independent of hTERT binding. Moreover, a mutation in the T motif (561) that drastically reduced nucleolar accumulation of YFP-NoLD protein in the U2OS and LM217 cells was similarly effective in the cells lacking hTR (Fig. 6B and Table II). These observations indicate that hTERT is not essential for the localization of hTERT to nucleoli.

Conclusions—We now show that hTERT is localized to the nucleolus when transiently expressed in human cells. Because both the hTR and hTERT subunits localize to this structure, we suggest that this localization may reflect a part of telomerase biogenesis such as the assembly of the hT and hTERT subunits into an RNP. However, it is possible that the targeting of hTERT to the nucleolus may have other functions such as the sequestration of telomerase from its telomeric target (30). The minimal fragment of hTERT defined by deletion analysis that mediates localization extends from amino acids 326 to 620. The most striking feature of this NoLD is that it encompasses all of the known hTERT binding elements of hTERT. Furthermore, mutating two of the three RNA-binding domains of hTERT reduced the localization of either the NoLD or the full-length hTERT to the nucleolus. Thus, initially appeared that the hT motif (561) that drastically reduced nucleolar accumulation of YFP-NoLD protein in the U2OS and LM217 cells was similarly effective in the cells lacking hTR (Fig. 6B and Table II). These observations indicate that hTERT is not essential for the localization of hTERT to nucleoli.
