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13. ABSTRACT (Maximum 200 Words)
A critical challenge in creating novel therapeutic agents in the treatment of breast cancer is the identification of cellular processes and molecular targets that differentiate normal and neoplastic tissue. One of the most consistent changes to occur n breast cancer is cellular immortalization through the upregulation of hTERT, which encodes the catalytic subunit of the telomerase ribonucleoprotein holoenzyme. Recently, we have identified the RNA binding domain of hTERT, and found that small substitutions to this region impede the binding of the hTR telomerase template RNA, and completely abrogate telomerase catalytic activity, thus defining and attractive region of hTERT that might be targeted for therapeutic targets. Interestingly, I have shown that this region of the protein also binds the endogenous inhibitor PinX1. I have demonstrated that the mechanism of PinX1 action may be distinct from inhibition of hTR binding, thus defining a separate mechanism for telomerase inhibition. We plan to identify molecules that bind hTERT and inhibit its action through displacement of hTR binding, to generate lead compounds that might be exploited for telomerase inhibition in cancer cells.

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Introduction

A basic and fundamental difference between cancer cells and normal somatic cells in the body is the capacity for unlimited cellular replication. With each cycle of cell division, chromosomes of somatic cells gradually shorten due to the inability of conventional DNA polymerases to replicate the extreme ends of linear DNA molecules (*1, 2*). While this shortening of telomeric DNA and the resulting genomic instability would otherwise limit cell proliferation, most cancer cells are able to overcome this proliferative barrier by the transcriptional upregulation of hTERT, the catalytic subunit of the telomerase enzyme. Telomerase extends telomere ends by catalyzing the addition of telomeric repeats complementary to its internal template bearing hTR RNA subunit thereby arresting telomere shortening and rendering cells immortal. In order to understand the functions of telomerase and gain insights into the mechanisms that could be targetted to inhibit telomerase, we performed an exhaustive scan of the previously uncharacterized amino and carboxyl terminal ends of hTERT that flank the catalytic core. Among the domains identified, we characterized the hTR binding domain of hTERT encompassing amino acids 326-620. We propose that this domain constitutes an important pharmacological target that could be exploited in breast cancer therapy.

Body

The objective of the proposed study has been to identify and validate regions of hTERT that can be pharmacologically targetted to inhibit the function of telomerase in breast cancer. While the catalytic core of telomerase had previously been characterized as having homology with reverse transcriptases and containing the seven conserved reverse transcriptases motifs (*3, 4*), the amino (N) and carboxyl (C) termini of the protein had been relatively uncharacterized.

To characterize these regions of hTERT, we performed an exhaustive scan by substitution mutagenesis where tandem six amino acid segments of the protein were substituted with the amino acid sequence NAAIRS (*5, 6*). Through this scan we identified eight *essential* regions of the protein that are critical for the catalytic activity of the hTERT protein, and thus amenable to pharmacological targetting. Of these, I showed that two N terminal *essential* regions along with the previously identified downstream T motif are involved in forming associations with the hTR template bearing RNA component of the telomerase holoenzyme. These regions mapping to amino acids +326-620 thus have been designated as the RNA binding domain (RBD) of hTERT (Appendix A – Figure 4).

Currently, I propose to search for molecules with affinity for the RBD of hTERT. I have previously validated the recombinant RBD to be functional and retain RNA binding properties when produced *in vitro* in rabbit reticulocyte lysate (Appendix C – Figure 3), and when produced in bacteria as a GST fusion protein, thus making it a valid

reagent in drug discovery screens. Recently, I have further delineated the function of this region and have attempted to purify it for drug discovery research.

Given our interest in developing molecules that might inhibit telomerase activity, I was interested in characterizing PinX1 – a protein recently reported to inhibit telomerase activity (*7*). In this aim, I first characterized the interaction of PinX1 with hTERT. To do this, I developed an *in vitro* assay where hTERT or portions thereof were produced and radio-labelled in rabbit reticulocyte lysate. These proteins were assayed for the ability to in interact with GST-PinX1 produced in bacteria. In this system, hTERT bound preferentially to GST-PinX1 in comparison to GST alone, and the control protein HDAC1 bound to both GST-PinX1 and GST at similar levels (Appendix B – Figure 1).

To map the area of hTERT that interacts with PinX1, I assessed the ability of PinX1 to bind to versions of hTERT that had a mutation in each of the eight recently elucidated domains of hTERT involved in enzyme catalysis. As all mutants were capable of binding PinX1, this approach was not successful in mapping the binding domain. Next, discrete regions of hTERT were assessed for their ability to interact with PinX1. From this analysis, it appeared that an N-terminal region of the protein overlapping with the RNA binding domain of hTERT was involved in the interaction with PinX1 (Appendix B – Figure 2).

From this analysis, it seemed that PinX1's mechanism of action was likely a result of hTR inhibition. In this case, its mechanism of action would corroborate hypothesis that inhibiting the hTR binding site represents an efficient mechanism to repress telomerase activity *in vivo*. However, I found that mutations to the RNA binding domain of hTERT at positions +386 and +512 and +563 that severely reduce hTR binding

(Appendix C – Figure 3), had no detectable affect on the ability of hTERT to bind PinX1 (Appendix B – Figure 3) defining hTR inhibition and PinX1 binding as separable phenomena. Thus we conclude that PinX1 inhibition of hTERT is likely to be distinct from blocking its RNA binding capability. This finding may be significant when screening for compounds that bind to the RBD, as it may be most desirable to pursue molecules that abrogate hTR binding while permitting the interaction with PinX1.

We propose a two-pronged approach to identifying molecules that bind the RBD of hTERT. First, we will conduct a phage display screen that will identify peptides that inhibit hTR binding. The effects of these inhibitory molecules can be easily assessed in cells that can express the peptides upon introduction of peptide coding sequences. The longer term goal of identifying lead inhibitory molecules, will be a part of a collaborative venture with the laboratory of Dr. Maurizio Pellechia. Through this effort, we plan to identify chemical compounds that are capable of interacting with the RBD of hTERT. In this case, ligand binding will be assessed by the ability to shift the NMR spectrum of the hTERT target region.

I have generated the protein target for these screens recombinantly in bacteria by expressing a fusion of GST with the RBD of hTERT. I have shown that this fusion protein is capable of binding radiolabelled hTR, and furthermore, that this protein is sensitive to a six amino acid substitution at position +512 known to diminish RNA binding (Appendix D). Unfortunately, this protein has proven be incredibly difficult to produce in abundance, and to purify. Furthermore, colleagues who have attempted similar phage display screens have alerted us to the fact that molecules often have a high affinity for the interface between the GST moiety and protein of interest, thus generating

a high level of 'background' in resulting screens. Due to these considerations, I have now re-engineered the RBD to contain a very small six amino acid histidine tag which can be purified through the use of a nickel column. This approach has the benefit of potentially superior purification and abrogates the use of a large bulky tag that can be problematic in ligand binding screens. Upon generation of this reagent, we will be able to proceed with our aims of identifying chemical and peptide ligands of hTERT capable of inhibiting telomerase activity.

Key Research Accomplishments (August 2002- August 2003)

- Demonstrated that the RNA binding region of hTERT made recombinantly in bacteria selectively binds to hTR in a manner that is sensitive to small sequence mutations
- Developed assay to analyze interaction between hTERT and PinX1
- Determined that interaction between hTERT and PinX1 is not dependent upon hTR RNA
- Determined that the RNA binding region of hTERT (aa. 326-620) overlaps with the PinX1 binding domain of the protein.
- Demonstrated that mutations to hTERT that compromise hTR binding do not appreciably affect PinX1 binding capabilities, suggestion that the mechanism of PinX1 inhibition does not involve inhibition of hTR binding.

Key Research Accomplishments (prior to August 2002)

- Identified amino acids 1127 to 1129 of hTERT as a *bona fide* C-DAT region that is involved in telomere regulation through a function that is distinct from nucleotide addition
- Characterized N and C terminal domains of hTERT with respect to involvement in: protein localization, hTR RNA binding, hTERT multimerization

Domain	Involved in hTR binding?	Involved in hTERT multimerization?	Involved in nuclear localization?
N-IA	No	No	
N-DAT	No	No	
N-IB	No	No	
N-II	Yes	No	
N-III	Yes	No	
N-IV	No	No	
C-I	No	No	
C-II	No	No	
C-III	No	No	
C-IV	No	No	
C-DAT	No	No	No

- Demonstrated ability for hTERT to multimerize *in vitro* independently of the hTR RNA subunit, and telomeric DNA
- Demonstrated that the isolated nucleolar localization domain binds the hTR RNA in vitro, and that RNA binding profile of NoLD mutants does not necessarily correlate with nucleolar localization
- Demonstrated that the isolated RNA binding domain of hTERT corresponding to amino acids 326-620 has hTR RNA binding activity when made in vitro in rabbit reticulocyte lysate, and when made as a recombinant GST protein in bacteria

Reportable Outcomes

Publications:

Poh, M., Boyer, M., Solan, A., Mitchell, S., Pedrotty, D., Banik, SSR., McKee, JA., Counter, CM., Niklason, LE. (Submitted). Blood Vessels Engineered from Elderly Human Cells.

McKee, JA., Banik, SSR., Boyer, MJ., Hamad, NM., Lawson, JH., Niklason, LE., Counter, CM. (2003). Human arteries engineered in vitro. EMBO Reports. 4, 1-6.

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Banik, SSR., Guo, C., Smith, AC., Margolis, SS., Richardson, DA., Tirado, CA., Counter, CM. (2002). C-terminal regions of the human telomerase catalytic subunit essential for in vivo enzyme activity. Mol Cell Biol, 22, 6234-6246.

Etheridge, KT., Banik, SSR., Armbruster, BN., Zhu, Y., Terns, RM., Terns, MP., Counter, CM. (2002). The nucleolar localization domain of the atalytic subunit of human telomerase. J Biol Chem, 277, 24764-24770.

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Armbruster, BN., Banik, SR., Guo, C., Smith, AC., Counter, CM. (2001). N-terminal domains of the human telomerase catalytic subunit required for enzyme activity in vivo. Mol Cell Biol, 21, 7775-7786.

Manuscripts:

Banik, SSR., Counter, CM. From Bread to Bedside: What budding yeast has taught us about the immortalization of cancer cells. In Nitiss, JL., Heitman, J. eds. Yeast as a tool in cancer research. Kluwer Academic Publishers. In preparation.

Abstacts Presented:

Human arteries engineered in vitro. Cold Spring Harbor Laboratory, Telomeres and Telomerase Meeting, 2003.

Validation of the RNA binding region of hTERT as a therapeutic target for cancer. Department of Defense, Breast Cancer Era of Hope Meeting, 2002.

C-terminal regions of the human telomerase catalytic subunit essential for in vivo and in vivo enzyme activity. Cold Spring Harbor Laboratory, Telomeres and Telomerase Meeting, 2001.

Degrees Obtained:

Ph.D., Duke University, Durham, NC, (September 1997 - December 2002)

Department of Pharmacology & Cancer Biology

Conclusions

To identify putative pharmacological targets of the hTERT protein subunit of telomerase, we performed an exhaustive scan of the previously uncharacterized amino and carboxyl terminal regions of the protein. Among domains that were identified as being essential for enzyme catalysis, I characterized two as being essential for the interaction with the hTR RNA subunit of telomerase. Recently I have also demonstrated that this region of hTERT interacts with PinX1, an endogenous protein inhibitor of telomerase; however the mechanism of PinX1 action appears to be distinct from blocking hTR binding.

Currently, we are planning two parallel screens to search for molecules to bind to the RNA binding site of hTERT. Through phage display and NMR technology, we hope to find peptides and small molecules that are capable of inhibiting telomerase, thus laying the groundwork for developing a novel therapeutic agent that may be used in the treatment of breast cancer.

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Appendices

Appendix A

Armbruster, BN., Banik, SR., Guo, C., Smith, AC., Counter, CM. (2001). N-terminal domains of the human telomerase catalytic subunit required for enzyme activity in vivo. Mol Cell Biol, 21, 7775-7786.

Appendix B

Characterization of the interaction between hTERT and PinX1

Appendix C

Etheridge, KT., Banik, SSR., Armbruster, BN., Zhu, Y., Terns, RM., Terns, MP., Counter, CM. (2002). The nucleolar localization domain of the catalytic subunit of human telomerase. J Biol Chem, 277, 24764-24770.

Appendix D

Recombinantly produced RBD is functional.

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N-Terminal Domains of the Human Telomerase Catalytic Subunit Required for Enzyme Activity in Vivo

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Most tumor cells depend upon activation of the ribonucleoprotein enzyme telomerase for telomere maintenance and continual proliferation. The catalytic activity of this enzyme can be reconstituted in vitro with the RNA (hTR) and catalytic (hTERT) subunits. However, catalytic activity alone is insufficient for the full in vivo function of the enzyme. In addition, the enzyme must localize to the nucleus, recognize chromosome ends, and orchestrate telomere elongation in a highly regulated fashion. To identify domains of hTERT involved in these biological functions, we introduced a panel of 90 N-terminal hTERT substitution mutants into telomerase-negative cells and assayed the resulting cells for catalytic activity and, as a marker of in vivo function, for cellular proliferation. We found four domains to be essential for in vitro and in vivo enzyme activity, two of which were required for hTR binding. These domains map to regions defined by sequence alignments and mutational analysis in yeast, indicating that the N terminus has also been functionally conserved throughout evolution. Additionally, we discovered a novel domain, DAT, that "dissociates activities of telomerase," where mutations left the enzyme catalytically active, but was unable to function in vivo. Since mutations in this domain had no measurable effect on hTERT homomultimerization, hTR binding, or nuclear targeting, we propose that this domain is involved in other aspects of in vivo telomere elongation. The discovery of these domains provides the first step in dissecting the biological functions of human telomerase, with the ultimate goal of targeting this enzyme for the treatment of human cancers.

A fundamental difference between normal somatic cells and malignant cells is the ability of the latter to proliferate beyond the normally defined set of cell divisions, through a process known as cellular immortalization. The ability of cancer cells to become immortal is linked to the replication of chromosome termini or telomeres. Telomeres are DNA-protein structures that protect chromosome ends from degradation and inappropriate recombination (8). The DNA portion of this structure in most eukaryotes is comprised of tandem repeats of a short G-rich sequence that extends past the complementary C strand, forming a 3′G-rich overhang that can adopt higher-ordered structures (8, 23). During DNA replication in normal human somatic cells, there is a loss of telomeric DNA, which eventually elicits a growth arrest signal in cultured cells termed senescence (26, 28, 55). If such a signal is disrupted, as it is in transformed cells, further telomere shortening eventually denudes chromosome ends of its protective DNA, leading to a period of crisis characterized by massive genomic instability and cell death (12, 55). Telomere loss may therefore serve as a protective mechanism to prevent sustained proliferation of abnormal cells that have a neoplastic predisposition.

Most cancer cells overcome the proliferative blockade of telomere shortening through activation of the normally dormant telomerase enzyme (3, 58). Human telomerase is a reverse transcriptase containing a ~127-kDa catalytic protein (hTERT) (27, 32, 41, 47) that reverse transcribes the template region of the associated RNA subunit (hTR) (18) onto the 3′ end of telomeric DNA, thereby elongating telomeres. Normally, somatic cells express only the hTR subunit (2, 18), but during tumorigenesis the hTERT gene is illegitimately activated, restoring telomerase activity, preventing further telomere shortening and thereby immortalizing cells (14, 33, 35, 41, 47, 48). hTERT is both required for the tumorigenic transformation of normal cells (16, 24, 54) and the continual proliferation of cancer cells (20, 25, 64). Since telomerase is activated in as many as ~85% of tumors but is absent in most normal tissues (3, 58), inhibition of hTERT could represent a specific means of targeting a broad range of cancers. Understanding how hTERT functions in human cells could be important for developing antitelomerase therapies.

Enzyme catalysis can be reconstituted in vitro with hTERT and hTR, suggesting that these subunits form the core of a more complex holoenzyme (4–7, 40, 43, 60, 61); however, the exact stochiometry of this core complex is uncertain. Biochemical purification of telomerase activity from the ciliate *Euplotes* suggests that the enzyme is composed of a single RNA, catalytic protein subunit, and associated protein (38). However, accumulating evidence suggests that telomerase may be a multimeric complex. For example, certain template mutations of the RNA were found to be copied in yeast and human cells only when a wild-type telomerase complex was present (51, 52, 60), and telomerase activity was immunoprecipitated with catalytically inactive hTERT fragments produced in telomerase-positive cells (7).

TERT proteins from a variety of organisms are defined by a large central catalytic domain, encompassing approximately one third to one half of the protein, which contains reverse transcriptase motifs essential for catalysis (46). C-terminal to

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this domain is a short highly divergent region, where the comparison of yeast and human proteins reveals little to no obvious sequence conservation or functional similarity (5, 7, 19; S. S. R. Banik et al., unpublished data). On the other hand, the N terminus of yeast telomerase contains four domains termed I, II, III, and the T-motif that are essential for yeast viability, with the latter two domains being necessary for RNA binding (9, 19, 63). In *Tetrahymena* spp., the N terminus is also essential for telomerase activity and a 321-amino-acid region encompassing the T-motif and domains II and III (as defined in yeast) can bind the telomerase RNA (36). Similarly, in vitro, deletion of the first 350 amino acids of human TERT abolishes telomerase activity, and a large 287-amino-acid N-terminal fragment of hTERT that maps to RNA-binding regions in the yeast and *Tetrahymena* protein has been shown to bind hTR (5, 7). More recently, an alignment of ~500 amino acids of the N terminus from an array of phylogenic TERT proteins identified five amino acids that are identical, clustering in three regions termed GQ, CP, and QFP, which overlap with yeast domains I, II, and III, respectively (63). Thus, the N terminus may contain evolutionarily conserved regions essential for RNA binding and telomerase activity.

Recent studies suggest that telomere elongation by hTERT involves more than the association with hTR or catalytic activity. Addition of a double hemagglutinin (HA) epitope tag to the C terminus of hTERT (hTERT-HA) results in a catalytically active enzyme that cannot maintain telomere length or immortalize cells in vivo (13, 49, 66). More recently, three different alanine substitution mutations in the N terminus of yeast catalytic subunit of telomerase, Est2p, have been found to dissociate catalytic from biological activity (19, 63). The biological function disrupted by these mutations is uncertain, since telomerase activity appears to be regulated at multiple levels in vivo. For example, the enzyme must localize to the nucleus to be functional, a process recently shown to be regulated during T-cell activation (39), possibly by phosphorylation or association of the protein 14-3-3 (39, 56). Telomerase is also targeted specifically to telomeres, and in yeast this process is mediated through a number of proteins (17, 22, 31, 50, 53, 65). Lastly, telomere elongation is known to be cell cycle regulated and tightly coupled to the synthesis of the complementary C strand (1, 15, 53). Biochemical analysis of in vitro reconstituted enzyme activity would not be expected to identify domains of TERT responsible for most of these other cellular functions. To elucidate the domains of hTERT required for such functions in human cells, we studied the consequence of mutations to hTERT in vivo. In addition to identifying domains essential for catalytic activity, we discovered a domain essential for another cellular function of telomerase. This DAT domain is dispensable for catalytic activity, but is required for in vivo telomerase function. This represents the first domain of hTERT linked to the biological regulation of telomerase.

MATERIALS AND METHODS

Plasmids. By using pairs of complementary oligonucleotides bearing the sequence AATGCTGCTATACGATCG (encoding for the sequence NAAIRS for the sense oligonucleotides) in place of the sequence encoding the six amino acids to be mutated (flanked on either side by 15 nucleotides complementary to native hTERT sequence), each NAAIRS substitution was introduced into either the *Eco*RI-*Mlu*I or the *Mlu*I-*Nco*I fragment of a N-terminal FLAG-tagged hTERT (FLAG-hTERT) by QuikChange site-directed mutagenesis (Stratagene). Accordingly, 90 separate oligonucleotide pairs were used to systematically substitute every six amino acids from the +2 position up to +547, with the exception of position +260. Mutated regions were sequenced to confirm correct substitution. To create retroviral constructs, all 90 of the mutated fragments were removed and cloned back into *Eco*RI-*Mlu*I or *Mlu*I-*Nco*I sites of full-length FLAG-hTERT cloned in the *Eco*RI-*Sal*I sites of the plasmid pBluescript SK(−) (Stratagene), after which the mutated open reading frame was excised and cloned into the *Eco*RI-*Sal*I sites in the retroviral vector pBabehygro (45). To create in vitro expression constructs, selected mutants were similarly extracted and cloned into the *Eco*RI-*Mlu*I or *Mlu*I-*Nco*I sites of an N- and a C-terminal FLAG-tagged hTERT cDNA (FLAG-hTERT-FLAG) that was inserted in the plasmid pCIneo (Promega). GST-pCIneo was made by digesting pGEX-4T1 (Amersham Pharmacia Biotech) with *Ssp*I and *Sal*I and then cloning this fragment into pCIneo digested with *Eco*RI (blunted with Mung bean nuclease) and *Sal*I. GST-hTERT-pCIneo (wild type; positions +50, +92, and +152) was produced by introducing FLAG-hTERT into GST-pCIneo with *Eco*RI and *Sal*I and then removing the FLAG sequence by PCR cloning with primers 5′-CGAATT CCAAACCGCCCCCTCCTTCCGCCAG and 5′-GTCCACGCGTCCTGCC CG. GST-hTERT-pCIneo (+386 and +512) were made by inserting *Mlu*I and *Sal*I fragments, digested from corresponding FLAG-hTERT-pBabehygro plasmids, into wild-type GST-hTERT-pCIneo cut with *Mlu*I and *Sal*I.

The hTR-expressing plasmid pBluescriptSK-hTR was created by inserting the *Eco*RI-digested hTR PCR product, generated by amplifying plasmid phTRA (10) with primers 5′-CGGAATTCGGGTTGCGGAGGG and 5′-CGGAATTC GCATGTGTGAGCCGAGTCCTGG into the same site downstream of the T7 promoter in pBluescript SK(−) (Stratagene).

Cell culture and apoptosis assays. The simian immunodeficiency virus (SV40) T/t-Ag transformed human embryonic kidney cell line HA5 (59) was infected at population doubling (pd) ~51 to 56 with the amphotropic retroviruses derived from the above-described pBabehygro constructs encoding each of the 90 NAAIRS mutant FLAG-hTERT cDNAs or, as controls, wild-type FLAG-hTERT or no insert, after which stable polyclonal populations were selected in media supplemented with 100 μg of hygromycin B (Sigma)/ml as previously described (13). A population doubling of 0 was arbitrarily assigned to the first confluent plate under selection. Cells were continually passaged at 1:4 or 1:8 under selection until either crisis or until the culture divided more than 2.5 times longer than vector control cell lines. Crisis was defined as the period when cultures failed to become confluent within 25 days and exhibited massive cell death.

For apoptosis studies, infected HA5 cell lines were split 1:4 or 1:8, and 3 days later the adherent cells were trypsinized and pooled with nonadherent cells from the media. These cells were washed twice in cold 1× phosphate-buffered saline (PBS) and stained with annexin V and propidium iodide according to manufacturer's instructions using the Annexin V-FITC Apoptosis Detection Kit II (Pharmingen). Flow analysis was performed at the Duke Comprehensive Cancer Center Flow Cytometry Shared Resource facility by using a FACSCaliber (Becton Dickinson).

hTERT mRNA detection, telomerase activity, and telomere length assays. For quantitative reverse transcription-PCR (RT-PCR), total RNA from each of the described infected HA5 cells was isolated with the RNAzol reagent according the manufacturer's instructions (Teltest), and 250 ng of RNA was RT-PCR amplified to detect either total hTERT or PBGD mRNA by using the LightCycler Telo-TAGGG hTERT Quantification Kit and LightCycler (Roche). hTERT signals were normalized to PBGD mRNA levels, and the number of hTERT transcript was determined by using a standard curve generated from RT-PCR of known concentrations of in vitro-transcribed hTERT mRNA, in accord with the manufacturer's instructions (Roche). Conversion to transcript per cell was determined based on the number of cell equivalents of RNA assayed.

To specifically detect endogenous or ectopic hTERT mRNA or the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA, the RNA described above was amplified by using semiquantitative RT-PCR as previously described (24) with primers specific for the following: endogenous hTERT, 5′-ACTCGACAC CGTGTCACCTA and 5′-GTGACAGGGCTGCTGGTGTC; ectopic hTERT, 5′-GACACACATTCCACAGGTCG and 5′-GACTCGACACCGTGTCACCT AC; or GAPDH, 5′-GAGAGACCCTCACTGCTG and 5′-GATGGTACATG ACAAGGTGC. Reaction products were resolved on 10% polyacrylamide gels, dried, and exposed to a phosphorimager screen.

To detect telomerase activity, lysates were isolated from infected HA5 cells at two different passages, protein concentration was measured by Bradford assay (Bio-Rad), lysates were diluted in the lysis buffer to a concentration of 0.1 μg/μl, and 0.2 μg was assayed for telomerase activity by using the telomeric repeat amplification protocol as previously described (34). As a negative control, duplicate reactions were heat treated at 85°C for 2 min to inactivate telomerase.

Reaction products were resolved on 10% polyacrylamide gels, dried, and exposed to a phosphorimager screen to quantitate enzyme activity as previously described (34).

Telomeres were visualized by Southern hybridizing 10 μg of *Hin*fI and *Rsa*I restriction enzyme-digested genomic DNA with the ^{32}P-labeled telomeric $(C_3TA_2)_3$ oligonucleotide exactly as previously described (12), with the exception that washes were performed with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Western blot and indirect immunofluorescence. To analyze mutant hTERT protein expression, 293T cells were transiently transfected with pCIneo or pCIneo-FLAG-hTERT-FLAG constructs by calcium phosphate transfection method (21). Cells were collected at ~48 h posttransfection and lysed in 1× PBS, 5 mM EDTA, 0.2% NP-40, 10% glycerol, 1 mM benzamidine, 1 μg of pepstatin A/ml, 1 μg of leupeptin/ml, 1.5 μg of aprotinin/ml, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM Na_3VO_4. The protein concentration was measured by Lowry assay (Bio-Rad), and 30 μg of soluble lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane (Millipore), and blocked with TBST (1× TBS [50 mM Tris-HCl, pH 7.4; 150 mM NaCl]–0.02% Tween 20)–5% milk. Blots were incubated with either anti-FLAG M2 mouse monoclonal antibody (Sigma) or anti-actin (C-2) mouse monoclonal antibody (Santa Cruz Biotechnology Inc.) and the goat anti-mouse immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology, Inc.) diluted in TBST–5% milk. Blots were washed three times for 6 min each time in 1× TBS or TBST, and protein was detected with ECL Reagent according to the manufacturer's protocol (Amersham Pharmacia Biotech).

Localization of hTERT proteins was visualized in the human osteosarcoma cell line, U2OS, by indirect immunofluorescence. A total of 2 μg of pCIneo, pCIneo-FLAG-hTERT-FLAG wild-type, or NAAIRS mutant +92 and +122 constructs were transiently transfected into U2OS cells by calcium phosphate and examined ~36 h posttransfection. Cells were fixed with 3% paraformaldehyde–2% sucrose, permeablized with 1× PBS–0.2% Triton X-100, and blocked with PBTN (1× PBS, 0.1% Triton X-100, 5% goat serum). Ectopic hTERT was detected by anti-FLAG M2 mouse monoclonal antibody recognized by a goat anti-mouse antibody conjugated with fluorescein isothiocyanate (Jackson ImmunoResearch) diluted in PBTN. Nuclei were stained with 2 μg of Hoechst 33258 (Sigma)/ml. Cells were examined at ×400 magnification on a Nikon Eclipse TE300 light microscope.

hTR-hTERT and hTERT-hTERT coimmunoprecipitations. hTR was expressed and ^{32}P labeled with the T7-coupled Maxiscript Kit (Ambion) by using 1 μg of linearized pBluescriptSK-hTR. Unincorporated nucleotides were removed by using a G-25 Minispin Column (Amersham Pharmacia Biotech). ^{35}S-labeled proteins were produced by using the T7 quick coupled TNT System (Promega) from plasmids pCIneo-FLAG-hTERT-FLAG; pCIneo-FLAG-hTERT-FLAG-NAAIRS +50, +152, +386, or +512; and pCMV-HDAC1-FLAG in the presence of 3 μl of hTR RNA.

For coimmunoprecipitations, 4.4 μg of the M2 anti-FLAG monoclonal antibody was prebound to 25 μl of GammaBind G-Sepharose (Amersham Pharmacia Biotech) in S-100 buffer (9 mM Tris, pH 7.5; 0.9 mM $MgCl_2$; 0.9 mM EGTA, pH 8; 1.5 mM dithiothreitol; 0.5% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}; 10% glycerol; 1 mM benzamidine; 0.1 mM phenylmethylsulfonyl fluoride) in the presence of blocking agents (100 ng of bovine serum albumin /ml, 100 ng of casein/ml, 100 ng of tRNA/ml, 250 ng of yeast total RNA/ml, 100 ng of glycogen/ml) as previously described with minor modifications (44). Coated beads were added to completed TNT reactions, diluted with S-100 buffer in a final volume of 750 μl, and incubated for 1 h at room temperature in the presence of 200 U of RNasin (Promega) and nonspecific blocking agents described above. The beads were washed three times with prechilled S-100 buffer, heated in SDS buffer, and resolved by SDS-PAGE.

For the immunoprecipitation of hTERT-hTERT complexes, wild-type and NAAIRS-substituted (+50, +152, +386, or +512) FLAG-hTERT-FLAG and N-terminal glutathione *S*-transferase (GST)-tagged hTERT were separately transcribed and translated as described above in the presence of 0.5 μl of $[^{35}S]$methionine and 1 μl of cold methionine, or 4 μl of $[^{35}S]$methionine, respectively, supplemented with 20 pmol of Ts oligonucleotide (34) and 1 μl of trace-labeled hTR RNA expressed in vitro with the Maxiscript Kit (Ambion) by using 0.17 μl of $[^{32}P]$UTP and 6 μM cold UTP. Reactions were incubated 30°C for 40 min, mixed with the appropriate reaction, and then incubated for an additional 60 min at 30°C. Reactions were immunoprecipitated with the anti-Flag M2 monoclonal antibody as described above. The reciprocal complex made with ^{35}S-labeled GST-hTERT and trace labeled FLAG-hTERT-FLAG was also immunoprecipitated with 1 μg of the Z-5 anti-GST antibody (Santa Cruz Biotechnology, Inc.). As controls, HDAC1-FLAG and GST were immunoprecipitated in the presence of GST-hTERT.

RESULTS

Identification of functional domains in the N terminus of hTERT by mutational analysis. To define domains essential for telomerase function, we generated a panel of 90 individual tandem NAAIRS substitution mutations within the N terminus of hTERT, beginning immediately after the initiating methionine and terminating at the conserved T-motif (46). NAAIRS substitution mutagenesis presumably has only minor effects on protein structure, since substitutions do not alter protein length and the NAAIRS sequence has the unique ability to adopt multiple structural conformations (62). Moreover, this mutagenesis approach has been successfully employed to map the pocket region of pRB (57), as well as locate C-terminal domains within hTERT (Banik et al., unpublished). The panel of NAAIRS substitution mutants was introduced into telomerase-negative HA5 cells by retroviral infection. HA5 cells are human embryonic kidney cells transformed with the SV40 T-Ag gene, which lack hTERT expression and lose telomeric DNA every cell division until they reach crisis and die (12, 59). The proliferative potential of these cells can therefore serve as a reliable indicator of the biological consequence of hTERT mutations, since stable expression of biologically active versions of hTERT restores telomerase activity, stabilizes telomere length, and immortalizes HA5 cells (see reference 13 and also below). The resulting HA5 infected cell lines were assayed for telomerase function in vitro by assessing telomerase enzyme activity and telomerase function in vivo by determining if the infected cells bypass crisis induced by telomere shortening.

To verify expression of hTERT mutants, we used quantitative RT-PCR to detect hTERT mRNA. This method was chosen because overexpression of hTERT by the retroviral promoter in HA5 cells produces undetectable levels of protein, as assessed by Western blotting with an anti-FLAG antibody (not shown). RNA was isolated from all 90 stably infected cell lines and RT-PCR amplified with primers specific for hTERT transcripts (Fig. 1A). Vector control-infected HA5 cells were found to have extremely low levels of hTERT mRNA, corresponding to ~1 transcript per 100 cells, which is >150-fold lower than that observed in tumor cell lines by quantitative SAGE analysis (37). Consistent with low hTERT expression, HA5 cells do not have readily detectable levels of telomerase activity, lose telomeric DNA, and fail to immortalize (12). Cell lines stably infected with FLAG-hTERT mutant constructs expressed hTERT mRNA at variable levels. However, in every case the expression was several orders of magnitude higher than vector cell lines when normalized with the housekeeping gene PBGD and was comparable to that detected in wild-type FLAG-hTERT-infected cells (Fig. 1A and Table 1).

Having confirmed that hTERT mutants were equivalently overexpressed, we next characterized each mutant cell line for in vitro telomerase activity and extended life span as a measure of in vivo telomerase function. As described in detail below, mutant hTERT proteins gave rise to four distinct phenotypes: nonessential (catalytically and biologically active), essential (catalytically and biologically inactive), slow growth (catalyti-

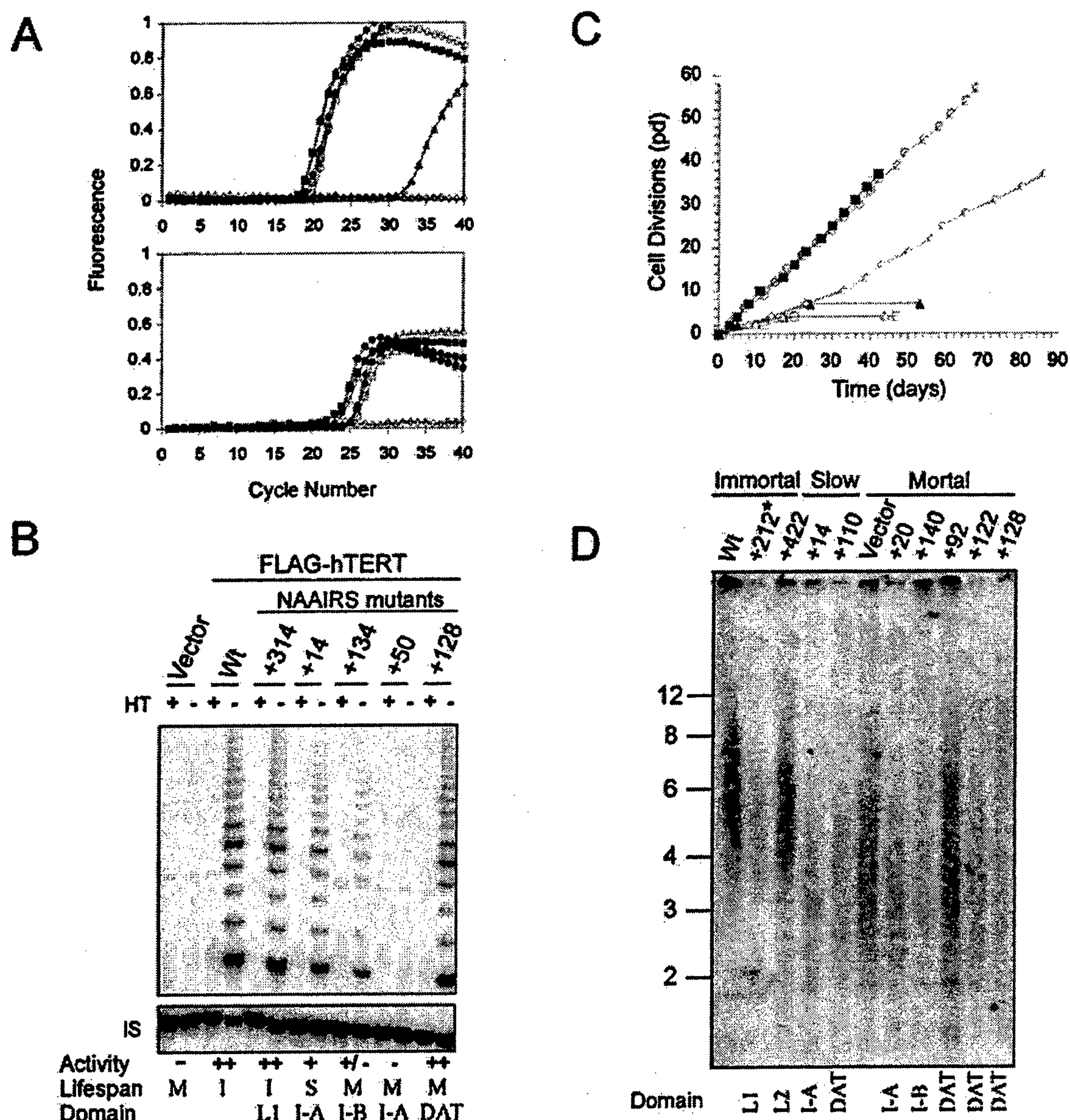

FIG. 1. Expression and telomerase activity of N-terminal hTERT mutants. (A) Total RNA was isolated from HA5 cell lines stably infected with vector (△), FLAG-hTERT (■, or FLAG-hTERT NAAIRS substitution mutants representative of nonessential (+212: ○), essential (+158, □), slow-growth (+110, ◆), and biologically essential (+128, ●) and RT-PCR amplified with primers specific for hTERT by quantitative, real-time RT-PCR. The amount of transcript detected by fluorescence with FRET probes is plotted in arbitrary units against each PCR cycle (top panel). The housekeeping PBGD transcript was similarly measured to verify equivalent RNA addition per reaction (bottom panel), while H_2O (◇) was assayed in both reactions as a negative control. (B) A total of 0.2 μg of lysate prepared from the described HA5 cell lines was assayed for telomerase activity by TRAP assay. As a control, a portion of the lysate was heat treated (HT) to inactivate telomerase prior to assaying. The internal standard (IS) served as a positive control for PCR amplification. Catalytic activity for each sample was normalized with the internal standard and is expressed as a percentage of wild-type FLAG-hTERT activity, indicated as follows: ++ (>60%), + (60 to 15%), +/− (<15%), and − (extremely low or no detectable activity). Domain refers to the location of the mutant, as described in the text. Life span (M, mortal; I, immortal; S, slow growth) as defined in the text. (C) Biologic activity of hTERT mutants was measured by serially passaging HA5 cell lines to determine whther cells entered crisis like vector or immortalized like wild-type hTERT. Representative clones are shown: vector (▲), FLAG-hTERT (■), +212 (○), +50 (□), +14 (◇), and +128 (△). (D) Telomere length of representative HA5 cells infected with NAAIRS mutants that result in an immortal, slow-growth, or finite life span was determined by releasing the terminal restriction fragments of genomic DNA isolated from the described cell lines at early passage (pd 2 to 3) with the restriction enzymes *Hin*fI and *Rsa*I. These fragments were resolved and detected by Southern hybridization with a telomeric probe. ✱, Sample +212 was underloaded. Domain refers to the location of the mutant, as described in the text.

cally active, biologically impaired), and biologically essential (catalytically active, biologically dead). Compilation of the different phenotypes with the respective mutation position revealed clustering along the primary amino acid sequence (Table 1; see also Fig. 3), implying distinct domains within the N terminus. Specifically, we defined four domains (I-A, I-B, II, and III) that are essential for catalytic activity, two nonessential or linker regions (L1 and L2), and one biologically essential

TABLE 1. hTERT expression and catalytic and biologic telomerase activity for the panel of N-terminal hTERT mutants

Domain and mutant[a]	hTERT expression (10^5)[b]	Telomerase in vitro activity[c]	Life span[d]
Vector	0.00001	–	M
Wild type	5	++	I
I-A			
+2	6	+	S
+8	4	–	M
+14	15	+	S
+20	2	–	M
+26	1	–	M
+32	3	++	S
+38	9	+/–	M
+44	2	+/–	M
+50[f]	7	–	M
+56	4	–	M
+62	2	++	I
DAT			
+68	3	++	M
+74	1	+	M
+80	8	+	M
+86	3	++	S/M
+92	2	++	M
+98	5	++	M
+104	7	++	S
+110	11	++	S
+116	1	++	S
+122	1	++	M
+128	6	++	M
I-B			
+134	1	+/–	M
+140	1	+/–	M
+146	1	+/–	M
+152[f]	10	–	M
+158	6	–	M
+164	3	–	M
+170	2	–	M
L1			
+176	1	++	I
+182	3	++	I
+188	0.4	++	I
+194	1	++	I
+200	1	++	I
+206	3	++	I
+212	0.4	++	I
+218	0.3	++	I
+224	1	++	I
+230	0.4	++	I
+236	3	++	I
+242	1	++	I
+248	1	++	I
+254	1	++	I
+266	1	++	I
+272	2	++	I
+278	2	++	I
+284	5	++	I
+290	3	++	I
+296	5	++	I
+302	5	++	I
+308	4	++	I
+314	5	++	I
+320	4	++	I
+326	6	+	I
+332	5	+	I
+338	4	++	I
+344	5	++	I
II			
+350	8	+/–	S
+356	3	–	M
+362	8	+/–	S
+368	3	++	I
+374	4	++	I
+380	6	+/–	M
+386[e]	9	–	M
+392	6	–	M
+398	20	+	S
+404	6	–	M
L2			
+410	7	++	I
+416	4	++	I
+422	5	++	I
+428	2	++	I
+434	6	++	I
+440	3	++	I
III			
+446	5	+/–	M
+452	3	–	M
+458	3	+/–	M
+464	6	–	M
+470	1	–	M
+476	1	–	M
+482	1	–	M
+488	2	+/–	M
+494	2	–	M
+500	3	++	I
+506	4	+/–	M
+512[e]	2	–	M
+518	5	+/–	M
+524	4	++	I
+530	4	–	M
+536	4	–	M
+542	4	–	M

[a] Each mutant is named after the starting position of the substitution in the native hTERT peptide sequence (i.e., +2 is [2]PRAPRC to [2]NAAIRS). Essential domains (I-A, I-B, II, and III), biologically essential domain (DAT), and nonessential linker regions (L1 and L2) are shown left of columns for mutant positions.

[b] Fold overexpression of hTERT transcripts compared to vector control after samples were normalized for RNA content with the transcript level of the housekeeping gene PBGD.

[c] In vitro telomerase activity for each mutant was determined by normalizing the activity to the internal standard and then expressed as a percentage of wild-type FLAG-hTERT activity as follows: ++ (>60%), + (60 to 15%), +/– (<15%), and – (extremely low or no detectable activity). At least two separate lysates were tested for each mutant.

[d] Polyclonal HA5 cells overexpressing FLAG-hTERT mutants were serially passaged to determine the life span. Lifespan was defined by similarity to growth of vector (negative control) and wild-type FLAG-hTERT (positive control) cell lines. Mutants that grew like vector eventually underwent crisis and are termed M (mortal), mutants that continually divide at a rate similar to that of the wild-type FLAG-hTERT are termed I (immortal), and mutants that continually divide at a slower rate compared to that of the wild type are termed S (slow growth).

[e] Mutants shown to have reduced hTR binding.

[f] Mutants shown to have wild-type hTR binding.

domain (Fig. 3). As discussed below, based on the ability of mutations within the biologically essential domain to separate in vivo and in vitro telomerase function, we have named this last domain the "dissociates activities of telomerase" (DAT) domain.

Linker regions are dispensable for telomerase activity. A total of 39 separate hTERT mutants were found to be phenotypically similar to wild-type hTERT, when expressed in HA5 cells. Lysates from the HA5 cells expressing these mutants contained high to moderate levels of catalytic activity, as measured by the ability of these extracts to elongate a single-stranded oligonucleotide with telomeric repeats (Fig. 1B and Table 1). It is formally possible that this activity was due to spurious activation of the endogenous hTERT gene, which would not be distinguished with the hTERT primers used to confirm FLAG-hTERT expression (Fig. 1A). To rule out this possibility, mRNA was isolated from representative cell lines containing NAAIRS substitutions in each of the nonessential regions at early passage, as well as at very late passage (a point after crisis of vector control cells), when the endogenous gene would be expected to be activated. This RNA was then RT-PCR amplified with primers specific for either endogenous or ectopic hTERT mRNA. Endogenous hTERT was found at neither early nor late (postcrisis) passage, despite clear expression of the ectopic TERT and a control housekeeping gene (Fig. 2), indicating that the observed telomerase activity in these mutants was a direct result of ectopic expression of the hTERT mutants.

Consistent with the high levels of activity, HA5 cell lines stably expressing each of these 39 mutants were, like wild-type hTERT-infected cells, able to bypass crisis and continued to proliferate in culture (Fig. 1C and Table 1). Additionally, cell

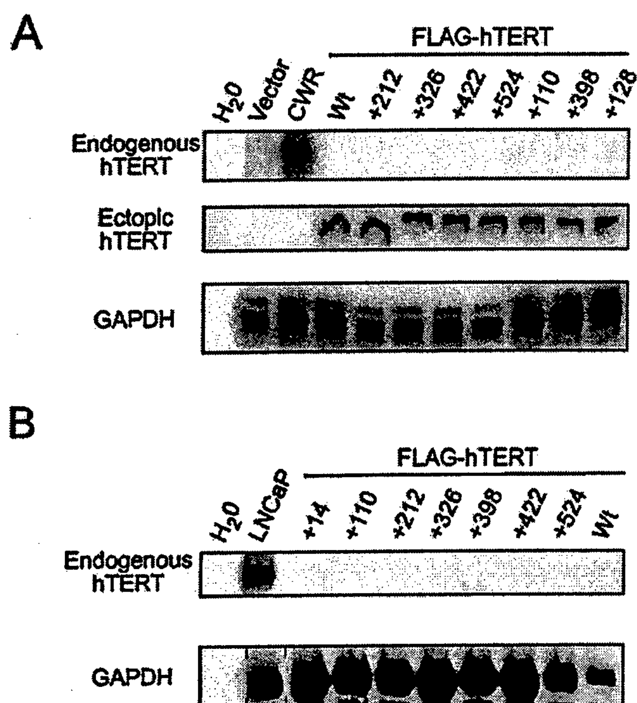

FIG. 2. Absence of endogenous hTERT expression in telomerase-positive HA5 cell lines. Total RNA collected from HA5 cells at either early passage (pd 2 to 3) (A) or at late passage (pd >39) (B) was analyzed by RT-PCR with primers specific for either endogenous or ectopic hTERT or GAPDH (control for RNA content). Results with nonessential (+212, +326, +422, and +524), slow-growth (+14, +110, and +398), and biologically essential (+128) mutants are shown. CWR and LNCaP are prostate cancer cell lines expressing endogenous hTERT and serve as positive controls for endogenous and a negative control for ectopic hTERT expression. A water sample is used to control for contaminating DNA in the reaction mix.

lines expressing representative mutants had larger telomeres (~6 kbp) compared to vector control HA5 cells (~4.5 kbp) at early passage (Fig. 1D). The two regions mapped by these mutants may serve as linkers, since these regions are, by all known biological criteria, nonessential for telomerase function and have little predicted secondary structure (Fig. 3).

N-terminal essential domains. Cell lysates from 34 independent hTERT mutants had extremely low or no detectable catalytic activity (Fig. 1B and Table 1). Like vector-infected cells, HA5 cells stably expressing these hTERT mutants lost telomeric DNA (Fig. 1D) and succumbed to crisis after undergoing a limited number of cell divisions (Fig. 1C and Table 1). Thus, loss of enzyme activity rendered these mutants biologically inactive, and hence these mutants define regions of hTERT that are essential. Mapping these essential mutants to hTERT sequence revealed a clustering in four regions (Fig. 3), which align with essential domains I, II, and III defined by mutational analysis in yeast (19), or homology blocks GQ, CP, and QFP (63). We note that, in humans, domain I is actually separated into two halves, which we term I-A and I-B, by a novel domain dispensable for telomerase in vitro enzyme activity (see below).

Since hTERT and hTR reconstitute a fully active enzyme in vitro, we reasoned that the absence of activity in essential domain mutants could be due to protein instability or loss in hTR interaction. To address the first possibility, we transiently overexpressed NAAIRS mutants from the four different essential domains in 293T cells to determine whether these mutants were produced at levels comparable to the wild type. Western blots indicated that there were no substantial differences in protein levels between the wild type and essential hTERT mutants (Fig. 4A) or noticeable degradation products (data not shown). Based on these findings, we propose that poor protein expression is not a major factor for reduction in catalytic activity of essential domain hTERT mutants. However, since hTERT protein is ectopically expressed at far higher levels transiently in 293T cells compared to stably in HA5 cells, we cannot rule out the possibility that a slight reduction in protein levels, not detected in 293T cells, could have an impact on telomerase activity when expressed in HA5 cells. The absence of telomerase activity in the described cell lines could also be argued to be due to low hTR levels. We discount this possibility since both telomerase-positive and -negative cell lines were derived from the same cells and because we assayed for telomerase activity in polyclonal populations, which are unlikely to have uniformly lower hTR expression in telomerase-negative cells compared to the similarly derived telomerase-positive cells.

Since a large deletion of the first 350 amino acids of hTERT abolishes both hTR binding and telomerase activity in vitro (7), we next addressed whether N-terminal essential domains are involved in hTR-binding. ^{32}P-radiolabeled hTR was incubated with ^{35}S-labeled double FLAG epitope-tagged hTERT generated in vitro and immunoprecipitated with an anti-FLAG an-

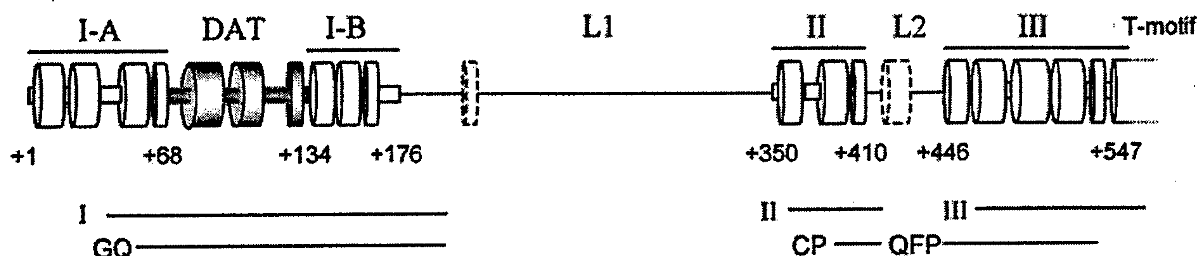

FIG. 3. Domain structure of the N terminus of hTERT. Secondary structure of the N terminus of hTERT as predicted by the Jprep2 program (http:jura.ebi.ac.uk:8888/) is shown, cylinders represent α-helices or β-sheets. Essential domains I-A, I-B, II, and III, as well as the T-motif, are denoted above structure prediction. Shaded regions denote the DAT domain; L1 and L2 define the nonessential linker regions. Two structured regions, outside of defined domains, are indicated by dashed lines. Essential domains I, II, and III found in the N terminus of Est2p (19) or conserved regions GQ, CP, and QFP identified in TERT proteins by alignment (63) are shown below structure prediction.

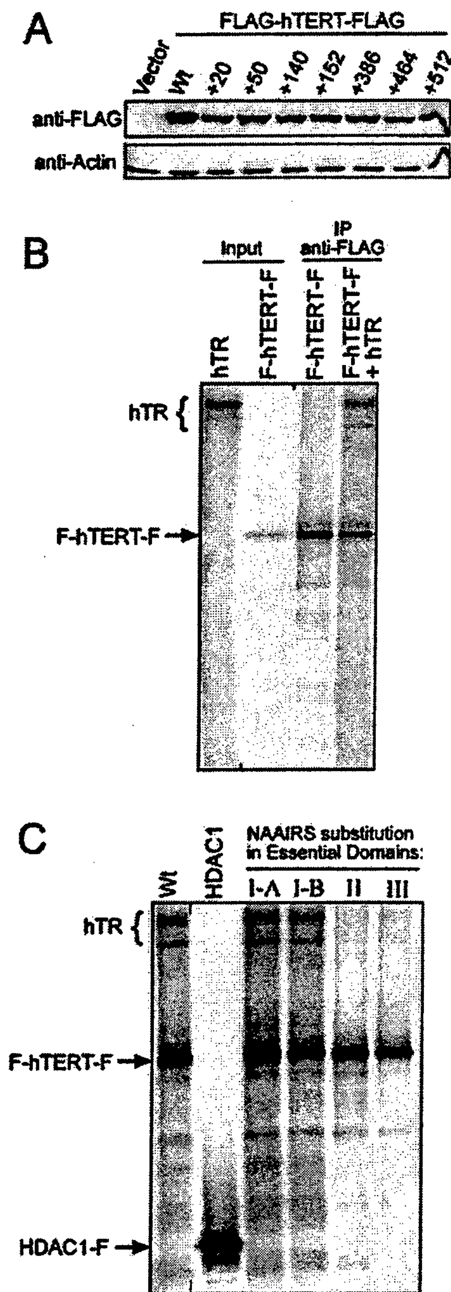

FIG. 4. Protein stability and hTR binding of mutants within essential domains of hTERT. (A) Lysates from 293T cells transiently transfected with FLAG-hTERT-FLAG, wild type, or the indicated NAAIRS mutants were resolved by SDS-PAGE and examined by anti-FLAG Western blotting. An anti-actin Western blot was used to ensure equal protein loading. (B) Binding of hTR with hTERT was examined in vitro by coimmunoprecipitating ^{35}S-labeled FLAG-hTERT-FLAG (F-hTERT-F) with purified ^{32}P-labeled hTR by using anti-FLAG antibodies. Immunoprecipitates were separated by SDS-PAGE and exposed to autoradiograph. Input hTR was diluted 1/1,000 and hTERT 1/10 for visualization. (C) Binding of hTR with FLAG-hTERT-FLAG protein containing NAAIRS substitutions (+50, +152, +386, and +512) in essential domains I-A, I-B, II, and III, respectively, was similarly examined. As a control for nonspecific interactions, HDAC1-FLAG (HDAC1-F) was immunoprecipitated in the presence of labeled hTR. The positions of F-hTERT-F, HDAC1-F, and hTR are indicated left of gel.

tibody to assess the hTR association (Fig. 4B). In this in vitro system, hTERT specifically interacted with hTR. The FLAG-tagged hTERT protein, but not the irrelevant FLAG-tagged protein HDAC1, coimmunoprecipitated hTR, despite the fact that HDAC1 was readily immunoprecipitated with the same antibody. Similarly, hTERT containing representative NAAIRS substitution in essential domains I-A and I-B (+50 and +152, respectively) also interacted with hTR, although these mutants are telomerase negative. However, immunoprecipitates of hTERT containing representative NAAIRS mutants within essential domains II and III (+386 and +512, respectively), which are essential for telomerase activity, showed a clearly visible two- to fourfold reduction in hTR binding. Since these mutants were expressed at levels equivalent to that of wild-type hTERT, we propose that domains II and III are critical for stable interaction between hTERT and hTR and that disruption of this interaction resulted in loss of enzyme activity.

hTERT mutants that only partially restore telomerase function. HA5 cells expressing nine different hTERT mutants were found to have impaired growth dynamics but nevertheless a greatly extended life span (Fig. 1C and Table 1). All but two (mutants +350 and +362) of these slow-growth mutant cell lines contained comparable levels of in vitro catalytic activity to cells infected with wild type or with nonessential mutants that had a similarly extended, if not immortal, life span (Fig. 1B and Table 1). Representative HA5 cell lines expressing these mutants did not have detectable levels of endogenous hTERT at early or late passage, indicating that enzyme activity was not due to activation of the endogenous hTERT gene (Fig. 2). Each of these mutant proteins could also be transiently expressed in 293T cells at levels equivalent to wild-type hTERT with no apparent proteolysis (Fig. 5A and data not shown). Although we cannot rule out that the proteins would behave identically when expressed in HA5 cells, the fact that most of these mutant are also highly telomerase positive argues against a loss of protein expression underlying the phenotypes of these mutants. Lastly, this slow-growth phenotype was reproducible. We infected HA5 cells again with three randomly chosen hTERT mutants that gave rise to the slow-growth phenotype (+32, +86, and +116); all had slow-growth, of which two continued to proliferate beyond crisis of vector control cells (not shown). Taken together, these data indicate that the slow-growth phenotype is directly related to the mutations in hTERT.

Slow-growth mutants lost substantial amounts of telomeric DNA at early passage; closely resembling telomere sizes found in telomerase-negative cells rather than in wild-type- or non-essential hTERT mutant-infected cell lines (Fig. 1D). The presence of shortened telomeres at early passage suggests the possibility that these cell populations teeter on the edge of extinction, with only a small fraction of cells having functional telomeres at any one time. One prediction of such a model is that the slow growth would result from increased cell death in the population. To test this prediction, we double stained late-passage HA5 cell cultures stably expressing three independent slow-growth mutants or, as controls, wild-type or nonessential mutants of hTERT, with annexin V, a marker of early apoptosis, and propidium iodide, an indicator of late-stage apoptosis (Fig. 5B). Two slow-growth mutants (+14 and +110) showed

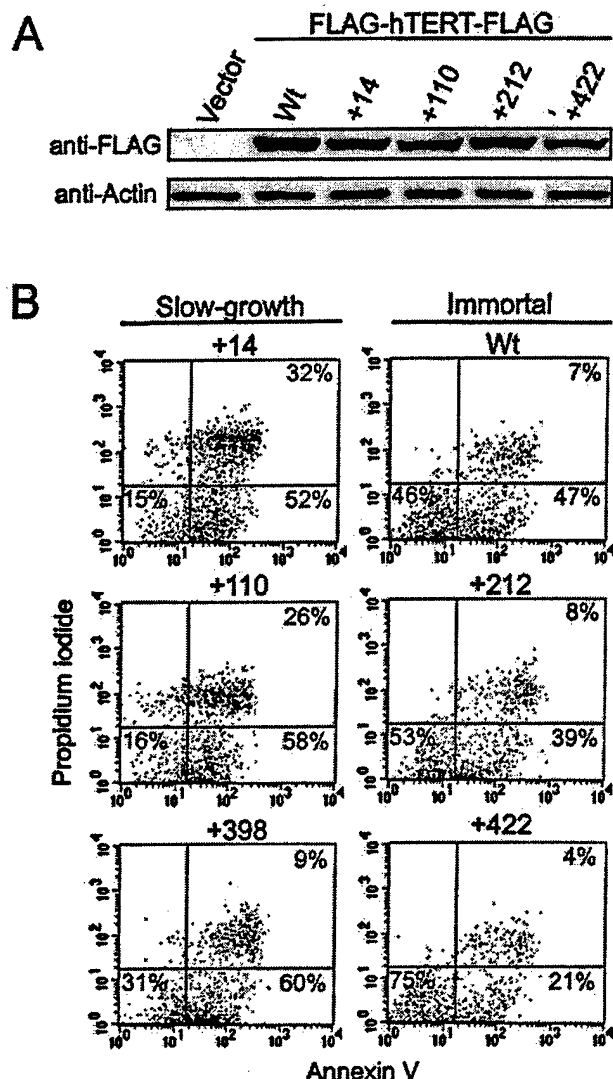

FIG. 5. Expression and cell viability of slow-growth hTERT mutants. (A) Anti-FLAG Western blot of slow-growth (+14 and +110) and nonessential (+212 and +422) hTERT mutants transiently expressed in 293T cells. Equal loading is shown by the anti-actin blot. (B) Viability of slow-growth and immortal HA5 cells was determined by flow cytometry of annexin V and propidium iodide double-stained cells. The percentages shown are averages for three independent experiments.

a >3-fold increase in the amount of double-stained, late-stage apoptotic cells, while the remaining mutant cell line (+398) exhibited higher levels of annexin V staining compared to normal immortalized HA5 cells. In every case, HA5 cells infected with slow-growth mutants had a lower amount of unstained, nonapoptotic cells than those infected with wild type or with nonessential hTERT mutants. Therefore, the apparent slow growth found in HA5 cells infected with the described hTERT mutants was a result of reduced viability of cells within the population.

The novel DAT domain is essential for in vivo telomerase function. A region of hTERT comprised of a series of eight NAAIRS substitution mutants were discovered to be dispensable for in vitro enzyme catalysis but essential for biological activity. Lysates from HA5 cells containing these mutants had high to moderate levels of in vitro telomerase activity (Fig. 1B and Table 1) and yet lost significant amounts of telomeric DNA (Fig. 1D) and were mortal (Fig. 1C and Table 1). Molecular characterization revealed that all of these mutants were overexpressed in HA5 cells (Fig. 1A) and representative mutants generated stable protein, at least when transiently expressed in 293T cells (Fig. 6A). Although it is formally possible that the stability of the same mutants may be much lower in HA5 cells, the fact that DAT domain mutations bestow high levels of telomerase activity in HA5 cells argues against this possibility. The biologically essential phenotype was also reproducible, since HA5 cells reinfected with three independent DAT domain mutants (+68, +92, and +128) demonstrated the same phenotype (not shown). Thus, mutations in the DAT domain disrupt functions distinct from those we have so far characterized by NAAIRS substitution analysis.

In vitro telomerase activity can be detected in lysates derived from human and yeast cells regardless of cell cycle progression

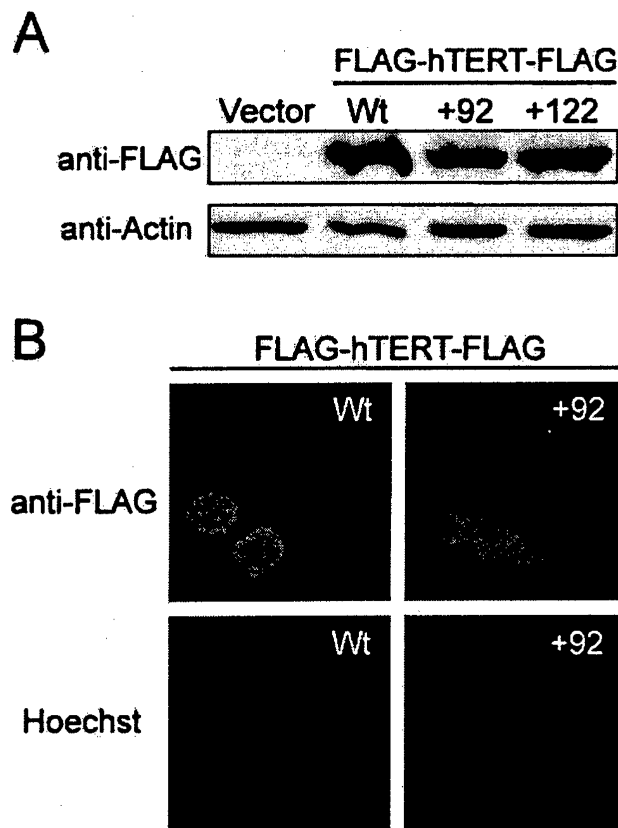

FIG. 6. Protein stability and nuclear localization of hTERT with mutations in the DAT domain. (A) Anti-FLAG Western blot of lysates from 293T cells transiently transfected with biologically essential hTERT mutants +92, +122, wild-type hTERT, or control vector. The anti-actin blot shows equal protein loading. (B) Subcellular localization of DAT domain mutants transiently expressed in U2OS cells by indirect immunofluorescence. Localization of FLAG-hTERT-FLAG was visualized with an anti-FLAG antibody recognized by a fluorescein isothiocyanate-conjugated secondary antibody (green). Hoechst was used to stain nuclei (blue).

(15, 30). However, telomeres are elongated in a cell cycle-dependent fashion in *Saccharomyces cerevisiae* (15), implying a regulation of the biological activity of telomerase. Indeed, binding of 14-3-3 proteins to hTERT has recently been reported to influence the subcellular localization of hTERT (56), raising the possibility that cytosolic-nuclear shuttling may be a regulatory mechanism for telomerase function in vivo. Loss of nuclear localization could leave a protein catalytically active but unable to reach its biological substrate. To therefore test whether the DAT domain is required for nuclear localization, an empty vector or one encoding double FLAG-tagged hTERT or representative DAT domain mutants NAAIRS +92 and +122 was transiently transfected into human U2OS cells and the resulting protein detected by indirect immunofluorescence with an anti-FLAG antibody. U2OS cells were chosen because they have a clearly defined nucleus and cytoplasm, which is ideal for monitoring nuclear localization. Wild-type hTERT was found predominantly in the nucleus of U2OS cells (Fig. 6B), although we did observe rare cells in which the signal was dispersed throughout the cell or localized to the cytosol (data not shown). Both DAT domain mutants displayed the same localization as the wild-type protein, being found predominantly in the nucleus, with some cells exhibiting cytosolic signals (Fig. 6B). We thus conclude that the biological dysfunction of the DAT domain mutants cannot be attributed to a failure in nuclear localization.

Finally, based on mounting evidence that hTERT may form homomeric complexes (7, 60), we investigated whether the defects in the DAT or essential domain I-A, I-B, II, and III mutants could be explained by the inability of these mutants to form higher-order complexes. We found that hTERT can indeed form homomeric complexes in vitro when expressed in rabbit reticulocyte lysates. This was determined by the ability to coimmunoprecipitate GST-hTERT and FLAG-hTERT-FLAG by using either an anti-FLAG antibody or an anti-GST antibody. This reaction could occur in the absence or presence of DNA substrate or the hTR subunit, indicating that multimerization is independent of these two parameters (Fig. 7A). The specificity of this interaction was demonstrated by the lack of an association of GST-hTERT with immunoprecipitated HDAC1-FLAG protein, as well as the inability of the FLAG-hTERT-FLAG protein to bind to immunoprecipitated GST (Fig. 7). We next tested whether mutations to any of the essential or DAT domains affected this interaction. GST- and FLAG-tagged hTERT containing representative mutants in the described domains were incubated and immunoprecipitated with an anti-FLAG antibody. In each case, both the GST- and FLAG-tagged protein were coimmunoprecipitated, arguing that the mutations did not affect multimerization, when expressed in rabbit reticulocyte lysates (Fig. 7B). Based on these in vitro experiments, the catalytic and biological defects of these mutants do not appear to be related to an impaired ability to form multimers, although we cannot exclude the possibility that the mutants may not multimerize in vivo.

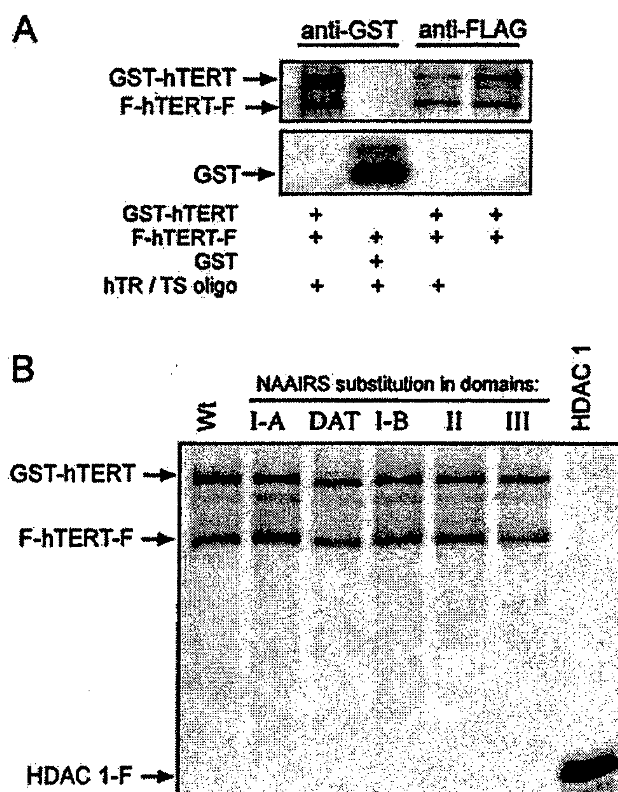

FIG. 7. Homomeric complex formation of essential and DAT domain hTERT mutants. (A) Immunoprecipitation of ^{35}S-labeled FLAG-hTERT-FLAG (F-hTERT-F) with either ^{35}S-labeled GST-hTERT or GST in the presence or absence of hTR and Ts oligonucleotide substrate with anti-GST or anti-FLAG antibodies as indicated. (B) ^{35}S-labeled GST-hTERT and F-hTERT-F, wild-type, or NAAIRS substitution in domains I-A, DAT, I-B, II, and III (+50, +92, +152, +386, and +512 mutants, respectively) were incubated together and immunoprecipitated with an anti-FLAG antibody to monitor protein association. As a control, an irrelevant FLAG-tagged protein (HDAC1-F) failed to coimmunoprecipitate GST-hTERT.

DISCUSSION

Functional conservation of N-terminal domains in evolutionarily diverse TERT proteins. We stably expressed a panel of tandem NAAIRS substitution mutants in telomerase-negative cells and screened for telomerase activity to identify functional domains in the N terminus of hTERT. NAAIRS substitution allows a large region of hTERT to be mutated with a reasonable number of changes, without altering protein length or causing large changes to secondary structure. The use of a cell-based screen allows mutants to be produced in a cellular environment containing factors that may be absent in vitro and allows each mutant to be simultaneously characterized for both in vitro and in vivo activity. The screen revealed four phenotypes for N-terminal mutants: essential (catalytically and biologically inactive), nonessential (catalytically and biologically active), biologically essential (catalytically active, but biologically dead), or slow growth (catalytically active, but biologically impaired). Mutants that give rise to the essential phenotype reside in one of four domains (I-A, I-B, II, and III) which were usually preceded by linker regions in which NAAIRS substitutions had minimal effects on the in vitro and in vivo functions of hTERT. We find that all four domains correspond to the yeast essential domains I, II, and III (19), as well as the similar domains GQ, CP, and QFP (63), defined by alignments of TERT proteins from evolutionarily diverse organisms (Fig. 3).

The domains that we defined as being essential in humans also appear to be conserved at the functional level with those of lower eukaryotes. Domain I in yeast (63) and humans (Fig. 3) is essential for long-term viability. Specific mutations in this region in these organisms, or in the ciliate *Tetrahymena* (36, 42), have little effect on RNA binding but result in a partial or complete loss of telomerase activity. In humans, the region defined as domain I is divided by the DAT domain and hence was termed domains I-A and I-B. Intriguingly, N-terminal hTERT mutants lacking the first 200 amino acids (including essential domains I-A and I-B and the DAT domain) are nonprocessive in vitro (7), whereas specific NAAIRS substitutions in the same region abrogate catalytic activity (Fig. 1B and Table 1). Perhaps the large deletion removes a portion of hTERT, which functions in an inhibitory manner when mutated by NAAIRS substitution. Nevertheless, both types of analysis define domains I-A and I-B as critical for proper enzyme function, as observed in lower eukaryotes.

Mutations in domains II and III were found to abolish telomerase activity and hTR binding and failed to rescue transformed human cells from crisis. In yeast, these domains are required for enzyme activity, telomere elongation, proliferation and, in the case of two mutants in domain III, RNA binding (19). Similarly, large fragments of TERT minimally encompassing these domains and the conserved T-motif can bind the RNA subunit in humans and *Tetrahymena* (7, 36). Like domain I, domains II and III appear to be functionally conserved.

We also found that, in addition to the sequence and functional conservation of the N-terminal domains of TERT, the most structured regions of this portion of hTERT mapped to the biologically defined domains of the protein, delineating these regions as important structural domains (Fig. 3). Therefore, with the exception of motifs unique to ciliates (9, 11, 42), the organization and function of the N-terminal domains appears to have remained intact throughout evolution. Thus, despite low sequence homology, the N terminus of TERT proteins do contain evolutionarily conserved functional domains.

The DAT domain in cellular regulation of telomerase. We identified a region termed the DAT domain defined by 11 contiguous mutants in which in vitro telomerase catalytic activity was dissociated from the ability of telomerase to function efficiently in vivo. Cells expressing DAT domain mutants either entered a period analogous to crisis observed in vector control cells or displayed less dramatic cell death, possibly representing a partial crisis. The latter phenotype, which we termed slow growth, was also found in cells expressing hTERT containing NAAIRS mutations in domain I-A (three-quarters of the slow-growth mutants mapped to either the I-A or the DAT domain). In one case, we found that a mutation in the DAT domain (mutant +86) could give rise to either a slow-growth or a mortal phenotype. Domains I-A, DAT, and I-B also form one continuous structured region that appears to have no obvious role in hTR binding or multimerization. Taken together, we speculate that the function of domains I-A and I-B may therefore be related to that of the DAT domain but that mutations to these two domains are more intrusive to biochemical activity.

Mutations in the DAT domain caused cell death, which was accompanied by a large decrease in telomere length, clearly demonstrating the loss of a novel in vivo telomerase function that is dispensable for biochemical enzyme catalysis in vitro. One aspect of telomere elongation that would not be represented in an in vitro assay for catalytic activity is posttranscriptional regulation of telomerase (15, 29, 39, 56). Recently, mutants have been isolated that affect hTERT entry into the nucleus, suggesting that cellular localization may be involved in coordinating hTERT-mediated telomere elongation (56). Although the DAT domain does not appear to contain any obvious nuclear localization sequence (NLS), nuclear localization could be mediated through a noncanonical NLS or a binding partner. However, we ruled out this possibility, since there were no noticeable defects in cellular localization of hTERT containing mutations in the DAT domain.

Although in vitro assayed telomerase activity purified from *Euplotes* consists of a single catalytic subunit (38), experiments from both yeast and human systems support the notion that the enzyme may function biologically in a complex containing more than one TERT and RNA subunit (7, 51, 52, 60). Disruption of this interaction could underlie the defect we observed in the DAT domain mutants. However, we find biochemically hTERT forms a homomeric complex in vitro, irrespective of mutations to the DAT domain. Thus, the biologically essential phenotype of the DAT domain cannot be ascribed to a failure of hTERT to multimerize.

Since mutations in the DAT domain neither grossly altered hTERT localization patterns nor homomultimerization, we speculate that this domain could instead be involved in recruitment of telomerase to telomeres. We note that mutations mapping to the corresponding DAT domain region in yeast Est2p had a similar biologically essential phenotype and that the proteins Est1p, Est3p, Cdc13p, and Ku are necessary for biological telomerase function and have been implicated in recruiting telomerase to telomeres in yeast (17, 22, 31, 50, 53). This raises the possibility that the hTERT DAT domain may interact with orthologs of these proteins. Alternatively, the DAT domain may participate in the coordination of 3′G-rich single-strand elongation by telomerase and lagging-strand synthesis of the C-rich strand (1, 15, 53).

Functional domains of hTERT. The ability of hTERT to elongate telomeres undoubtedly requires complex and precise regulation involving nuclear import, substrate recognition, and coordinated synthesis of the C strand. Since it is not feasible to reconstitute this complex process in vitro, we employed intact human cells to scan hTERT for regions that will further our understanding of these important biologically defined functions. The identification of the biologically essential DAT domain has clearly demonstrated the utility of this approach and represents a definitive step in elucidating the regulation of telomerase function in vivo. Lastly, since the inhibition of telomerase has been shown to prevent cancer cell lines from forming tumors in vivo, all of the essential domains that we identified in hTERT may represent suitable pharmacological targets for the treatment of human cancers.

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Appendix B

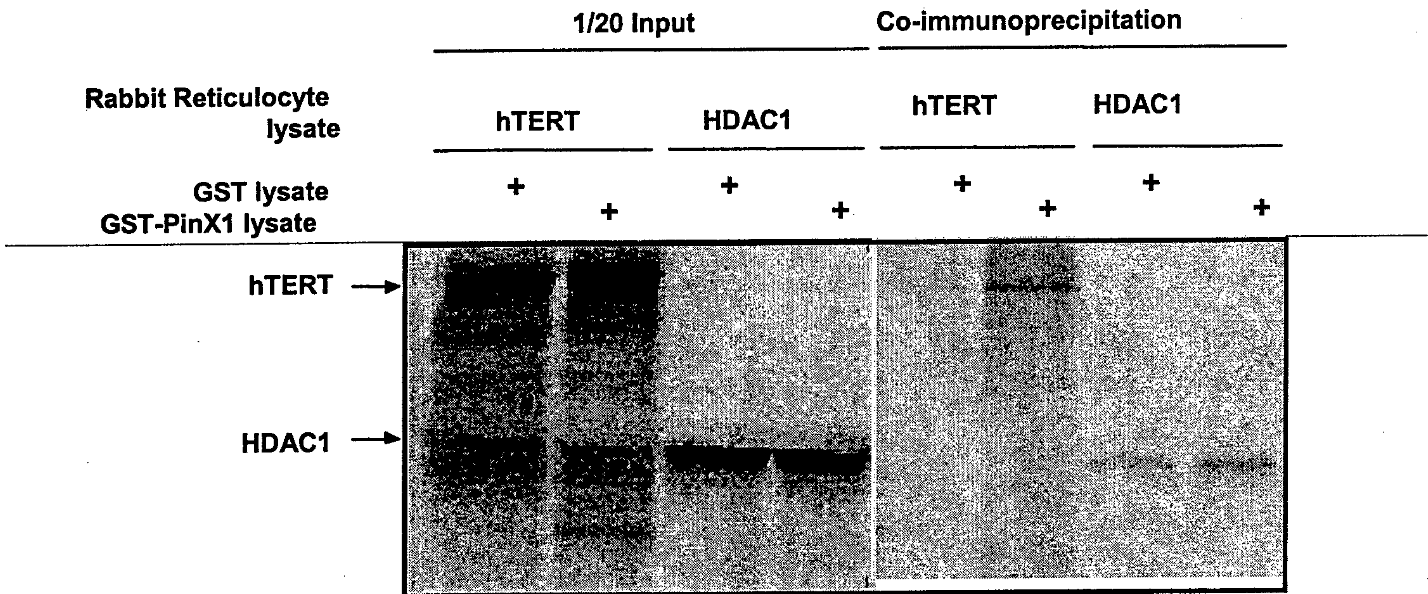

Figure 1 – hTERT interacts with GST-PinX1 *in vitro*

hTERT or the control protein HDAC were produced and ^{35}S labelled in rabbit reticulocyte lysate and incubated with GST-PinX1 or GST alone. Proteins were immunoprecipitated using an anti-GST antibody.

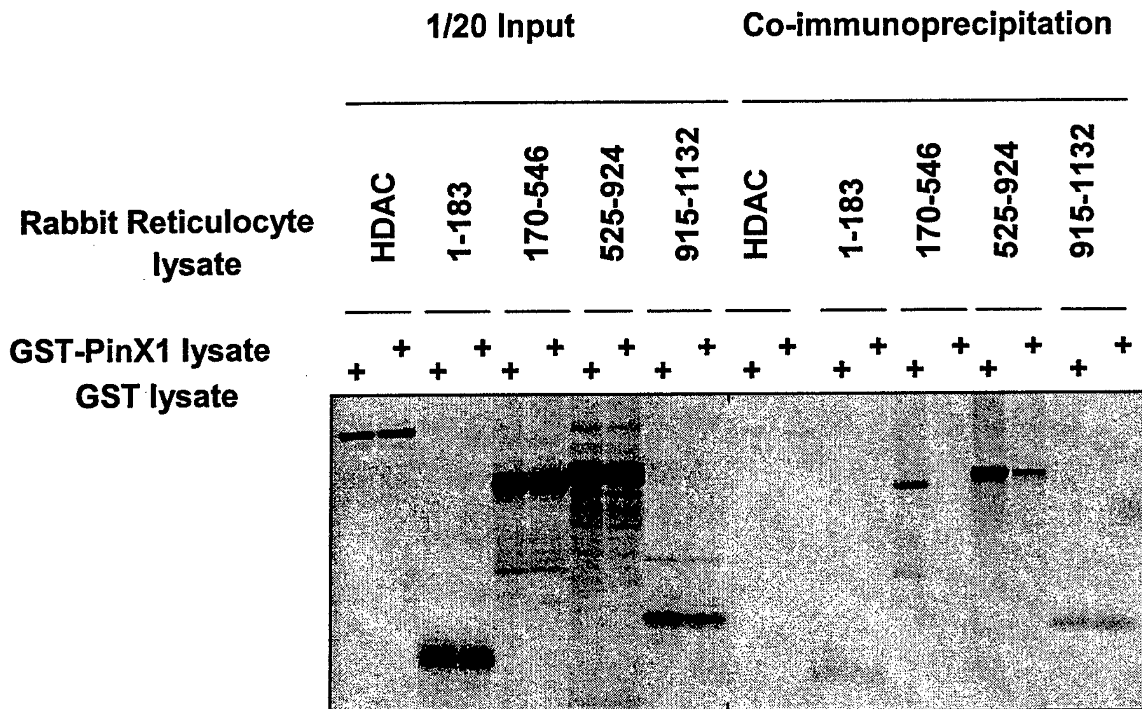

Figure 2 – N terminal regions of hTERT interact with GST-PinX1 *in vitro*

hTERT or portions thereof were produced and ^{35}S labelled in rabbit reticulocyte lysate and incubated with GST-PinX1 or GST alone. Proteins were immunoprecipitated using an anti-GST antibody. HDAC was used as a control.

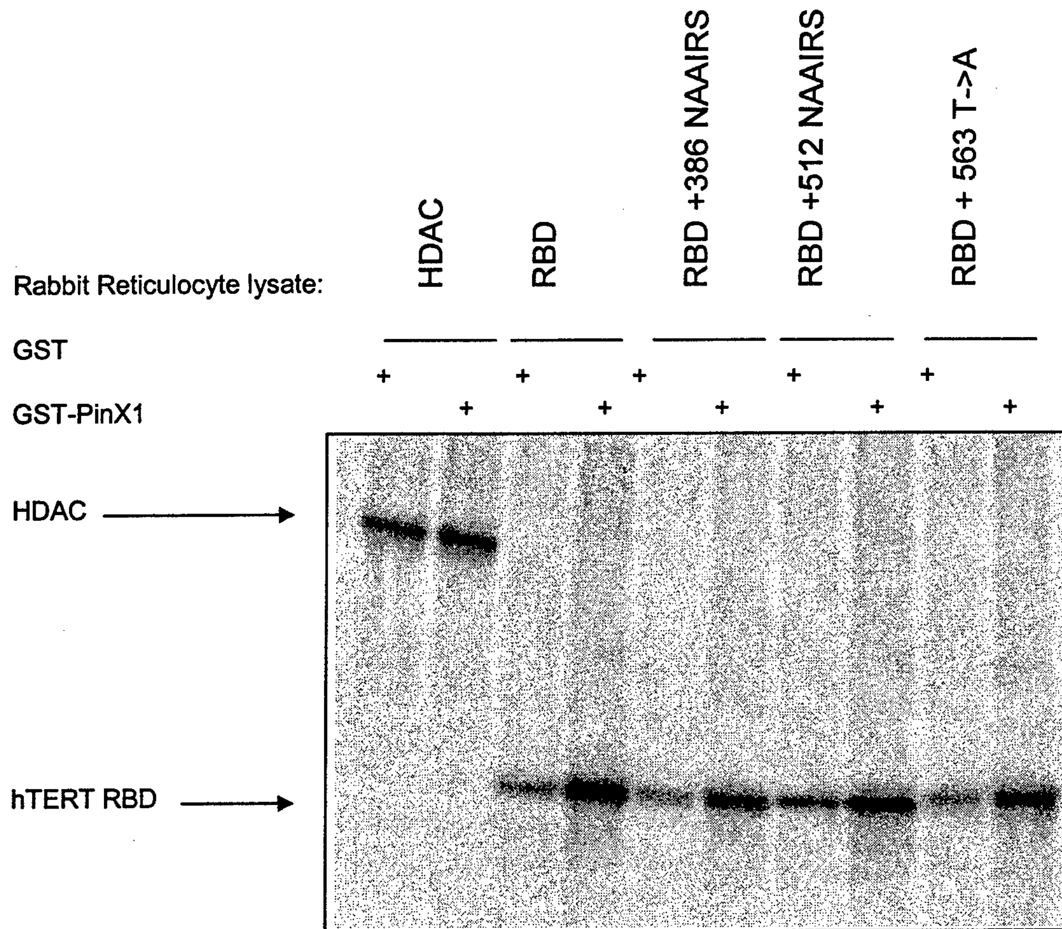

Figure 3 – RBD mutants of hTERT bind to PinX1

Wild type and mutant RBD were produced and ^{35}S labelled in rabbit reticulocyte lysate and incubated with GST-PinX1 or GST alone. Proteins were immunoprecipitated using an anti-GST antibody. HDAC was used as a control.

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

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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 nucleolus, a site of assembly of ribosomes as well as other ribonucleoproteins (RNPs). We therefore tested whether the hTERT component is also found in the nucleolus, where it could complex with the hTR RNA to form a functional enzyme. We report here that hTERT does indeed localize to the nucleolus, 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 nucleolus 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 nucleolus supports the premise that the assembly of telomerase occurs in the nucleolus.

Telomerase is a reverse transcriptase ribonucleoprotein (RNP)[1] complex composed of a reverse transcriptase catalytic protein subunit (TERT) that copies a template region of an accompanying 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 DAT (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 TERT 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 nucleolus in vertebrate systems. The nucleolus 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 rRNA processing and modification within the nucleolus (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 nucleolus 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 spliceosomal 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 nucleolus, 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 nucleolus, 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

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[1] The abbreviations used are: RNP, ribonucleoprotein; TERT, telomerase reverse transcriptase catalytic protein subunit; TR, telomerase RNA subunit; hTR, human TR; hTERT, human TERT; YFP, yellow fluorescent protein; NoLD, nucleolar localization domain; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.

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nucleoli 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 Tissue Type Collection) and LM217 (38) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Plasmids—pYFP-hTERT, hTERT-II_{386}, or TERT-III_{512} were made by subcloning the *Eco*RI/*Sal*I FLAG-hTERT cDNA either in the wild type format or with a six-amino acid (NAAIRS) substitution of the sequence [386]YWGMRP in domain II or [512]MSVRGC 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′-GGAATTCGCCACCATGGACTACAAAGACGATGACGAC-AAGCTGAGGCCCAGCCTG-3′ and 5′-TGCGGTCGACTCATCTGGAC-GTCAGCAG, digesting the product with *Eco*RI/*Sal*I, and cloning the fragment into the same sites in plasmid pEYFP-C1, which had a modified polylinker to accommodate this reading frame or the same sites of pCI-neo (Promega) for *in vitro* synthesis. The same six amino acids described above beginning at positions 386 and/or 512 were substituted with the sequence NAAIRS, and/or amino acid Phe^{561} in the T motif was mutated to Ala in the NoLD fragment by site-directed mutagenesis as described previously (20), and the resultant products were confirmed to be correct by direct sequencing. These products were again subcloned into the *Eco*RI/*Sal*I sites of the modified pEYFP-C1 plasmid or pCI-neo. hTERT deletion fragments were generated by PCR with the primers 5′-TCCCCGCGGAAGCTTGCACCATGCCGCGCGCTCCCCGC-3′ and 5′-ATTGGATCCATGGCCTGAGTGGCAGCGCC-3′ (1–183), 5′-CGCG-GAAGCTTGCTACCAGCTCGGCGCTGCC-3′ and 5′-ATTGGATCCAT-CCAGTGCAGGAACTTGGC-3′ (170–546), 5′-CGCGGAAGCTTGCCC-AGGGGTTGGCTGTGTT-3′ and 5′-CCGCTCGAGTCGACTAGTGGGC-CGGCATCTGAAC-3′ (523–924), and 5′-CGCGGAAGCTTGCGATGAT-TTCTTGTTGGTG-3′ and 5′-ATTGGATCCATGTCGACTCAGTCCAG-GATGGTC-3′ (867–1132), confirmed to be correct by direct sequencing, digested with *Hin*dIII/*Sal*I or *Hin*dIII/*Bam*HI, and cloned into the same sites in pEYFP-C1.

hTR-hTERT Co-immunoprecipitation—As described previously (20), hTR was transcribed and ^{32}P-labeled with the T7-coupled Maxiscript kit (Ambion) using 1 μg of linearized pBluescriptSK-hTR plasmid (20), after which unincorporated nucleotides were removed by 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 ^{35}S-labeled in the presence of 1 μl of hTR using the T7 quick coupled TNT system (Promega). As a negative control, FLAG-HDAC1 was expressed from the T7 promoter of pCMV-HDAC1. 10 μl of anti-FLAG M2-agarose (Sigma) was used for immunoprecipitation after preblocking with 100 ng/ml bovine serum albumin, 100 ng/ml casein, 100 ng/ml tRNA, 250 ng/ml yeast total RNA, and 100 ng/ml glycogen in PBS supplemented with 1.5 mM dithiothreitol, 0.5% CHAPS, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride. 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 Telo*TAGGG* 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 WI38 VA13/2RA cells grown on coverslips coated with 100 mg/ml poly-D-lysine, M_r > 300,000 (Sigma). Cells were transiently transfected with the above described plasmids encoding the appropriate YFP fusion protein using either the FuGENE 6 (Roche Molecular Biochemicals) or LipofectAMINE 2000 (Invitrogen) reagents according to the manufacturers' protocols. After 48 h, live U2OS or LM217 cells were observed under PBS at ×400 magnification on a Zeiss Axioskop fluorescence microscope. VA13 and HeLa cells were fixed in 4% formaldehyde in PBS

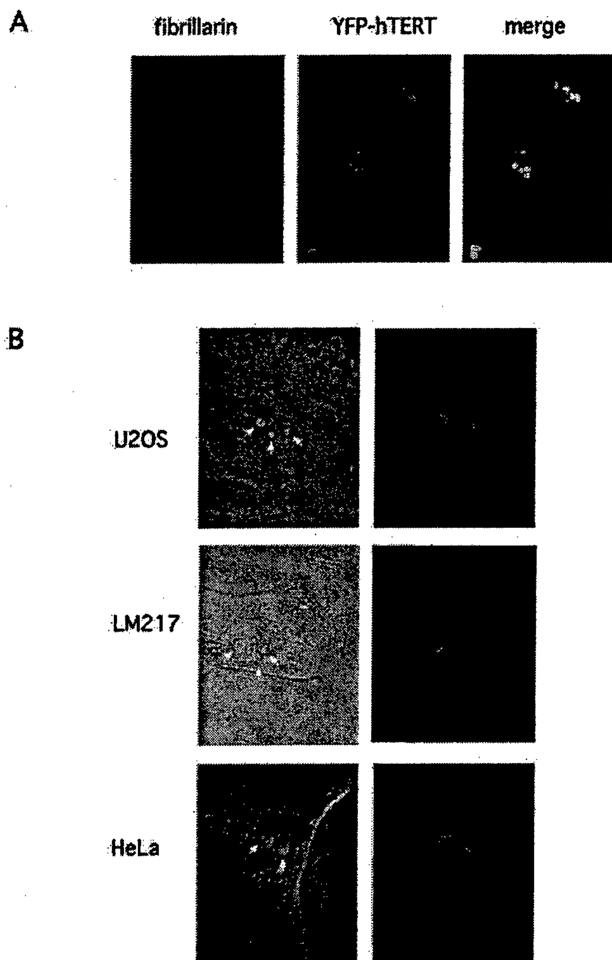


FIG. 1. **hTERT is found in the nucleolus.** *A*, an example of U2OS cells transiently expressing YFP-hTERT and stained with an anti-fibrillarin antibody to detect the nucleolar protein fibrillarin (*left*) or viewed as a fluorescence image to detect YFP-hTERT (*middle*) or a merge (*right*) of both 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.

TABLE I
Subnuclear localization of YFP-tagged hTERT

The described YFP fusion proteins were transiently expressed in the human 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.

YFP fusion	Cell type	Nucleolus[a]
		%
hTERT	U2OS	82
	LM217	96
	HeLa	93
	VA13	80
hTERT-II_{+386}	U2OS	57
hTERT-III_{+512}	U2OS	73

[a] 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+nucloplasm; hence, only cells with such staining were scored as positive for being in the nucleolus.

for 10 min at room temperature, washed twice with PBS, mounted, and observed at ×630 magnification on a Zeiss Axiovert S100 inverted fluorescence microscope. To visualize fibrillarin, U2OS cells transfected with hTERT expression constructs were similarly fixed and then per-

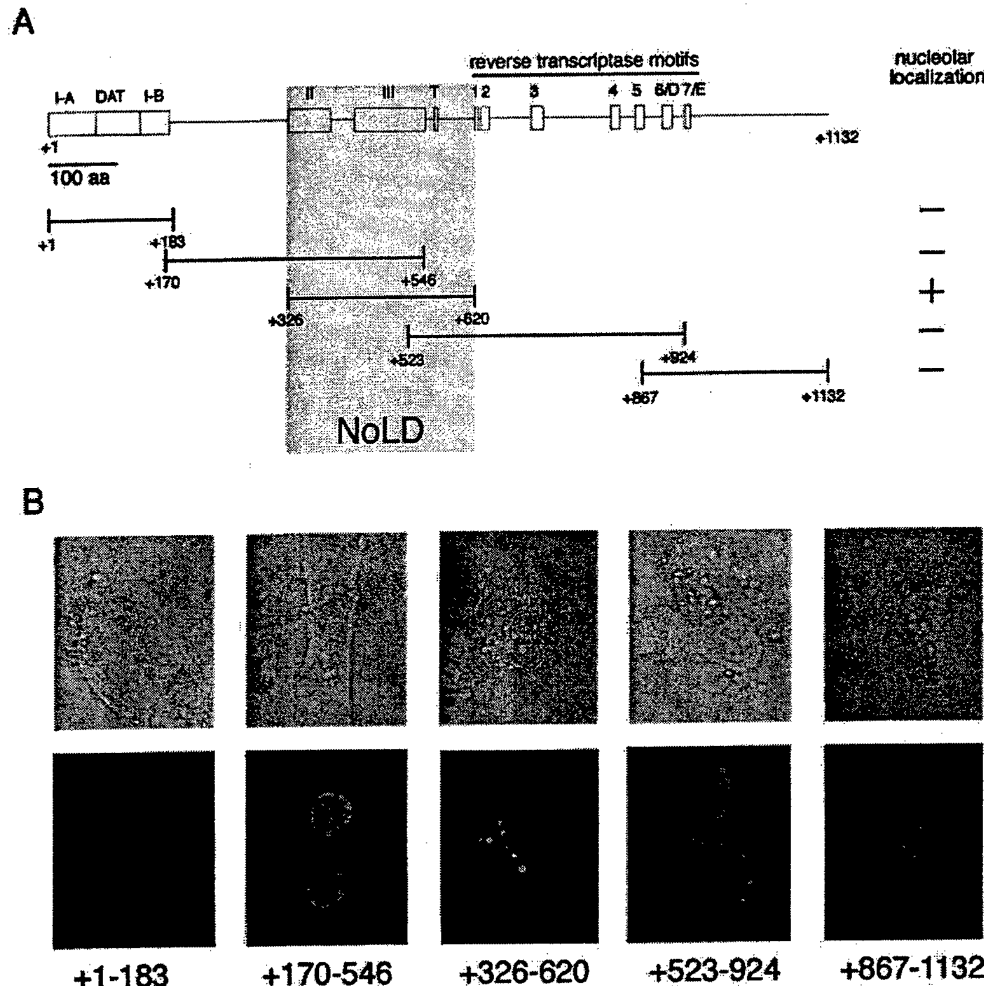


FIG. 2. **Deletion mapping identifies the nucleolar localization domain of TERT.** *A*, a scale diagram representing the known regions of hTERT (refer to text for description) is shown above a line diagram depicting the size and position of hTERT fragments that were fused to YFP. The presence (+) or 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*. *aa*, 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.

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

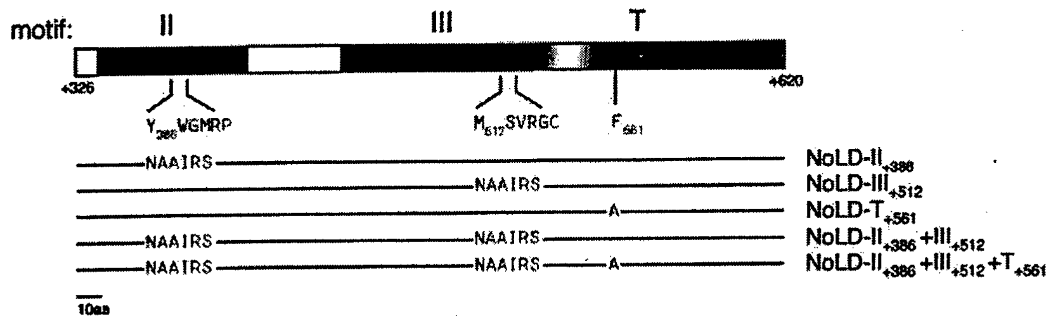
TABLE II
Subnuclear localization of YFP-tagged NoLD proteins

The described YFP fusion proteins were transiently expressed in the human U2OS, LM217, HeLa, or VA13 cells, after which a minimum of 100 cells with nuclear YFP protein were scored for nucleolus staining and expressed as a percent based on the total number of cells observed.

YFP fusion[a]	Cell type	Nucleolus[b]
		%
NoLD	U2OS	100
	LM217	99
	HeLa	95
	VA13	97
NoLD-II_{+386}	U2OS	29
	LM217	35
NoLD-III_{+512}	U2OS	70
	LM217	74
NoLD-T_{+561}	U2OS	25
	LM217	34
	HeLa	5
	VA13	6
NoLD-II_{+386} + III_{+512}	U2OS	26
NoLD-II_{+386} + III_{+512} + T_{+561}	U2OS	20

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

A



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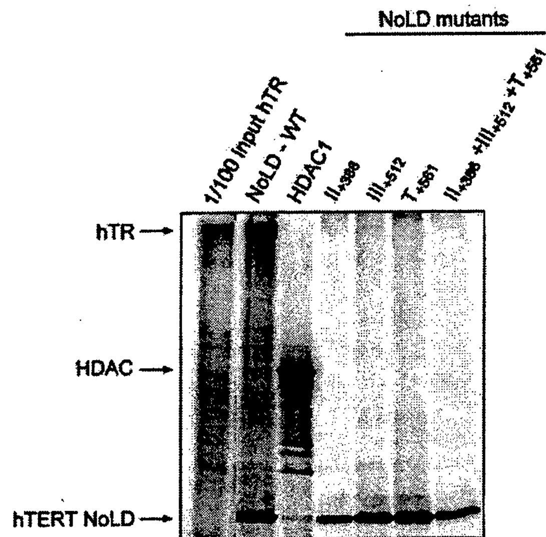

FIG. 3. **Mutations in the nucleolar localization domain that disrupt hTR binding.** *A*, *top*, a scale representation of the NoLD, the minimum fragment (326–620) found to be required for nucleolar localization. *Black boxes* denote the most conserved regions, domains II and III and the T motif, whereas *shading* denotes bordering sequences that are less conserved, and *white shading* identifies regions known to be dispensable for enzyme activity. The positions and sequence of specific amino acids mutated are shown below. *Bottom*, diagram depicting the size and position of mutations of the NoLD fragments fused to YFP. On the *right* are listed the names of the mutants. *aa*, amino acids. *B*, wild type (*WT*) and the described mutants of the FLAG-tagged NoLD domain of hTERT were ^{35}S-labeled and made in the presence of ^{32}P-labeled hTR. The irrelevant protein HDAC-1 was used to assess the level of non-specific RNA binding. RNA-protein complexes were immunoprecipitated by an anti-FLAG antibody, and products were resolved on an SDS-PAGE. RNA input is represented as 1/100 of the post-immunoprecipitation lysate.

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. 1*B* 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. 2*A*). 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. 2*B*). 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. 2*B* and Table II), indicating that this fragment contains sequences sufficient for nucleolar targeting. We found that the overlapping fragment 170–546,

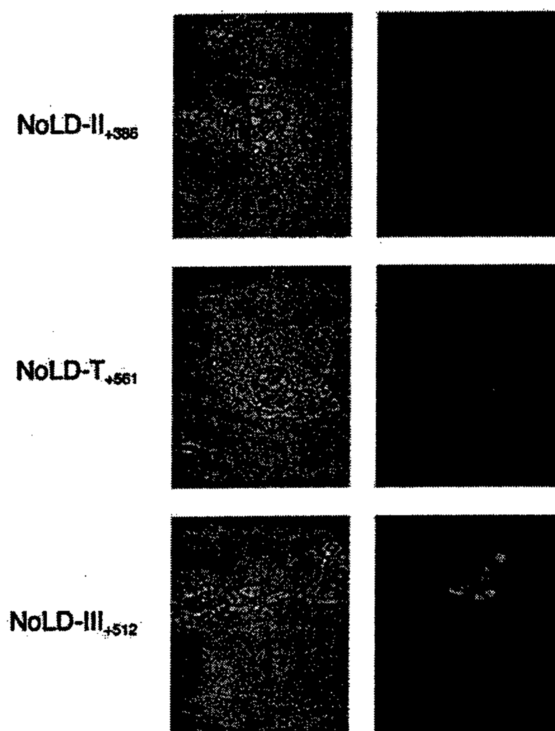

FIG. 4. **Mutations in domain II and the T motif but not domain III disrupt nucleolar localization.** Examples of a U2OS cell transiently expressing the YFP-tagged NoLD region of hTERT containing the 386 mutation in domain II (*top*), the 561 mutation in the T motif (*middle*), or the 512 mutation in domain III (*bottom*) are shown as phase contrast images (*left*) to visualize nucleoli or as fluorescent images (*right*) to visualize the YFP-tagged proteins.

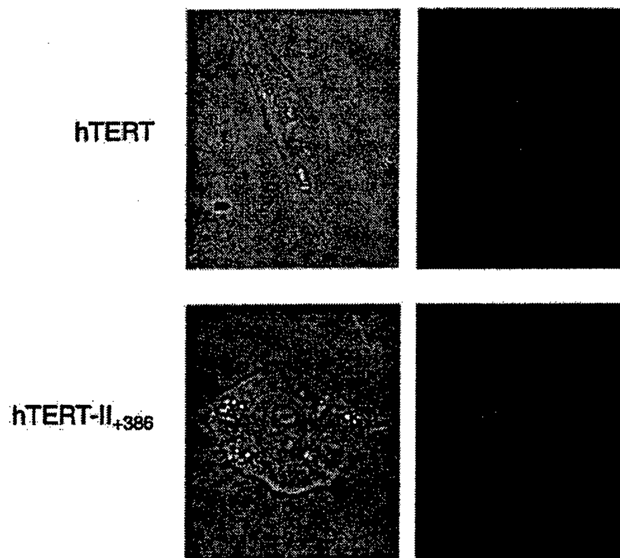

FIG. 5. **Mutations in domain II inhibit the accumulation of full-length hTERT in the nucleolus.** An example of a U2OS cell transiently expressing the YFP-tagged hTERT either in the absence (*top*) or presence (*bottom*) of the 386 mutation in domain II is shown as phase contrast images (*left*) to visualize nucleoli or as fluorescent images (*right*) to visualize the YFP-tagged protein.

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 NAAIRS) 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. 3*A*). We then confirmed that the NoLD fragment harboring mutations in domains II and III and the T motif abolished hTR binding *in vitro*. Specifically, ^{35}S-labeled FLAG-tagged NoLD fragments, either in the wild type format or with the 386, 512, or the 561 mutations, were incubated with ^{32}P-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. 3*B*). 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. 3*B* and Ref. 20), greatly reduced the nucleolar localization of the NoLD fragment. In >400 cells scored, the mutated YPF-NoLD fragment was found in the nucleolus only one-third of the time, a 3-fold drop compared with the wild type NoLD fragment. Similarly, a mutation in the T motif (561) crippled the ability of the NoLD fragment to localize to the nucleolus by 4-fold. Interestingly, mutating domain III (512) had little effect on the nucleolar localization of the NoLD fragment (Fig. 4 and Table II). We discount the possibility that the localization of these three mutant NoLD proteins was dependent upon cell type, because all of these proteins were similarly localized when expressed in LM217 or HeLa cells (Table II). Thus, disrupting two of the three hTR-binding domains altered the nucleolar targeting by the NoLD, 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-

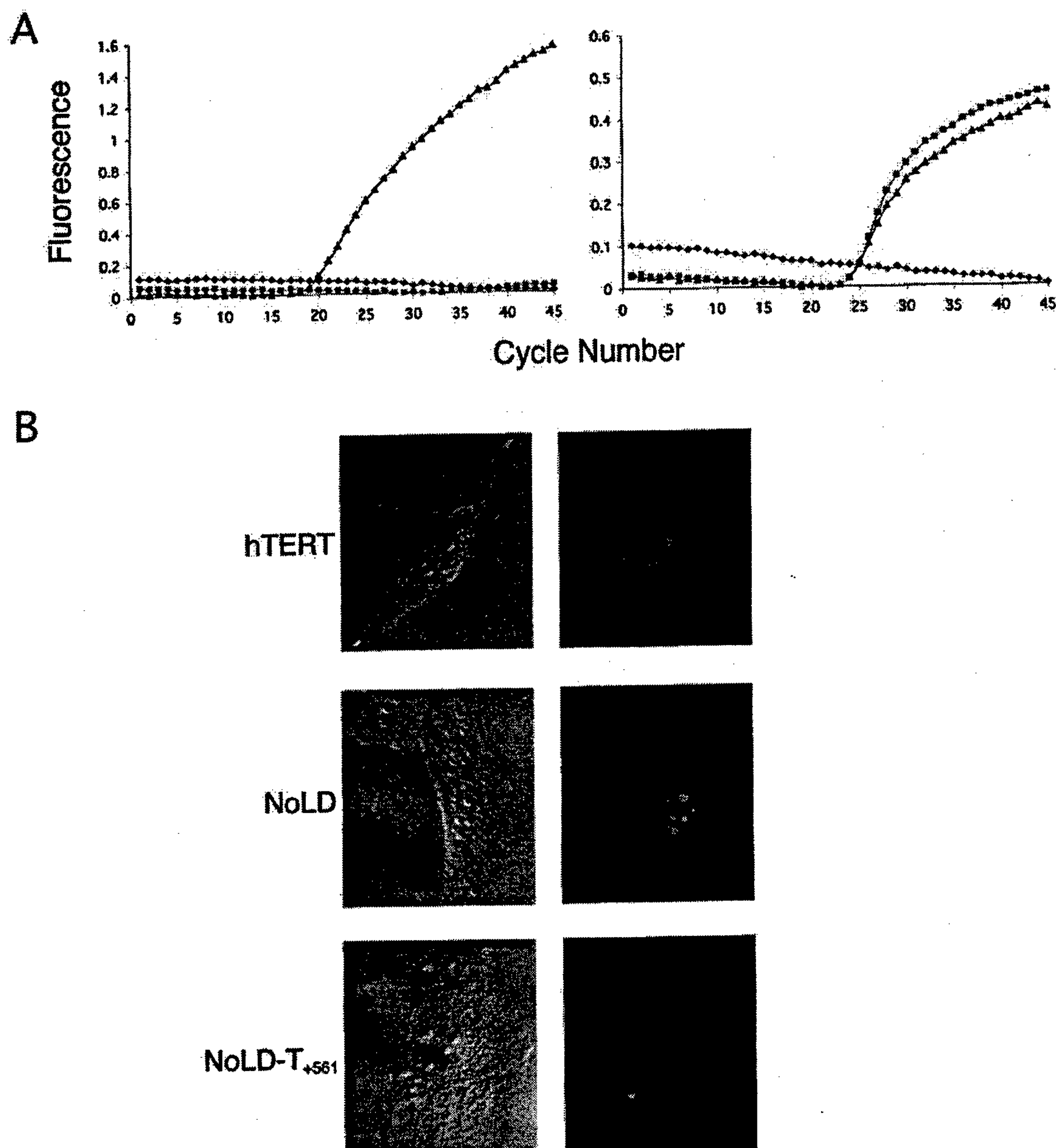

FIG. 6. **hTERT localizes to the nucleolus in cells that do not express hTR.** *A*, total RNA isolated from telomerase-negative VA13 cells (■) or, as a positive control, telomerase-positive CWR22 prostate cancer cells (▲) or, as a negative control, water (♦) was reverse transcribed and PCR amplified with primers specific for hTR (*left*) or, as a control for RNA content, the mRNA of the housekeeping gene *PBGD* (*right*). The amount of transcript detected by fluorescence with fluorescence resonance energy transfer (FRET) probes is plotted in arbitrary units against each PCR cycle. *B*, examples of a VA13 cell transiently expressing the YFP-tagged hTERT (*top*) or the NoLD fragment either in the absence (*middle*) or presence (*bottom*) of the 561 mutation in the T motif are shown as differential interference contrast images (*left*) to visualize nucleoli or as fluorescent images (*right*) to visualize the YFP-tagged proteins.

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. 3*A*). 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.

hTERT Nucleolar Localization Is Independent of hTR Binding—We have shown that the nucleolar localization domain of hTERT maps to the region of the protein linked to hTR binding (Fig. 2) and that mutations in two known hTR-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 hTR recruiting hTERT to the nucleolus via the Box H/ACA motif. However, one mutation that abolished all measurable association of hTERT with hTR (Fig. 3*B*) 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, hTR binding may be essential for hTERT localization, but the mutation in domain III does not disrupt hTR 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 hTR transcript is not present in these cells despite being readily detected in telomerase-positive cells (Fig. 6*A*). If hTR 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 hTR 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. 6*B* and Table II). These observations indicate that hTR 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 hTR 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 hTR 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, it initially appeared that the hTR served as a bridge, tethering hTERT protein to the Box H/ACA motif for nucleolar localization. However, we also found that a mutation in hTERT that disrupts hTR binding did not grossly affect the ability of the protein to accumulate in the nucleolus and that hTERT is found in the nucleolus of cells lacking hTR. The simplest interpretation of these data is that the NoLD can target hTERT to the nucleolus in a manner that is independent of hTR binding. Thus, the targeting or retention of hTERT within the nucleolus may involve other factors that interact specifically with domain II and the T motif within the NoLD of hTERT.

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Appendix D

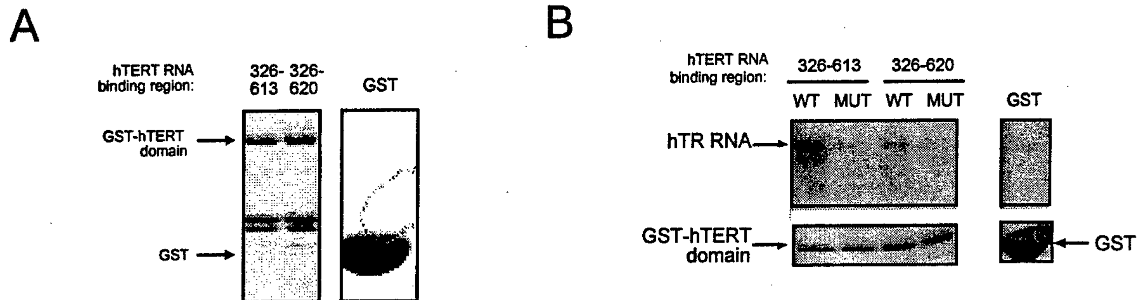

Figure 1 - Recombinant RBD of hTERT is functional

A) Two GST fusion protein fragments corresponding to the hTR binding domain of hTERT were produced in bacteria and immunoprecipitated with an anti-GST antibody and resolved by SDS PAGE. The fragments differ only by the length of their C-terminus.

B) The same two protein fragments (WT) or versions containing a mutation at position +512 (MUT) were incubated in the presence of ^{32}P labelled hTR, immunoprecipitated as before and Resolved by SDS-PAGE. GST serves as a negative control in A and B.