DYNAMIC TRAFFICKING OF TELOMERASE COMPONENTS

by

REBECCA LEAH TOMLINSON

(Under the Direction of Michael Terns)

ABSTRACT

Telomerase is the ribonucleoprotein (RNP) enzyme that synthesizes telomeric repeats at chromosomal termini. Telomerase is inactive in the majority of adult human somatic cells, and the telomeres in these cells grow progressively shorter over an individual's lifetime. In contrast, telomerase activity is present in over 90% of cancer cells, and maintenance of telomere length in these cells is critical for their sustained proliferative capacity. Telomerase is thus a promising target for anti-cancer treatments. An understanding of human telomerase regulation would greatly aid in the development of such treatment strategies. Of particular importance is how and where within the cell telomerase is assembled from its component parts and how access of telomerase to telomeres is governed.

Here, we present evidence that telomerase accesses to its substrate telomere only during a precise window of the cell cycle and suggest that S phase-specific telomere synthesis is regulated at the level of subnuclear trafficking of the telomerase RNP. The main components of telomerase, telomerase RNA (TR) and telomerase reverse transcriptase (TERT) are housed in separate nuclear foci throughout the majority of the cell cycle. During S phase a dynamic shift in localization of each

component occurs. Telomerase transits through two subnuclear structures, Cajal bodies (CBs) and nucleoli, before reaching its functional destination, the telomere. Further, we find that the trafficking of telomerase to CBs and telomeres is closely linked to biogenesis of the enzyme.

We also present evidence that CBs serve as sites of telomerase biogenesis. TR localizes to CBs in cells that are telomerase-positive, but not those that are telomerase-negative. Localization of TR to CBs is dependent on TERT expression, and ectopically expressed TERT can be found within CBs. Injection studies in *Xenopus* oocytes show that TR and TERT are assembled coincident with CB localization of the RNA.

However, integration into CBs does not seem to be an important step in the trafficking of telomerase in mouse cells, as mTR is housed within foci separate from CBs throughout the cell cycle. Similar to the occurrences in human cells, mTR is recruited to subsets of telomeres specifically during S phase, making this a potentially useful system to study factors that mediate this recruitment.

INDEX WORDS: Telomerase, Telomerase RNA (TR), Telomerase Reverse Transcriptase (TERT), Telomere, Cajal body, Nucleolus, Intranuclear trafficking, Telomerase regulation

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REBECCA LEAH TOMLINSON

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REBECCA LEAH TOMLINSON

Major Professor: Michael Terns

Committee: Mark Farmer
Sidney Kushner
Michael McEachern
Walter Schmidt
Rebecca Terns

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2007

DEDICATION

To my parents, Don and Mary, for their constant faith in me and my abilities.

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TABLE OF CONTENTS

Page

Chapter 1

Introduction and Literature Review

Telomeres

The origins of telomere biology lie in the early cytogenetic studies of Barbara McClintock (1). Studying the fate of broken chromosomes in maize, she discovered that ruptured chromosomes would re-fuse with other (available) ends. Natural chromosome ends were immune to this end-to-end fusion, leading McClintock to postulate that the extreme termini of eukaryotic chromosomes had special protective properties.

Telomeres, nucleoprotein structures found at the physical ends of linear eukaryotic chromosomes, do indeed function as a protective cap, preventing DNA repair activities (like end-to-end fusions) at chromosomal termini. Telomeres were first characterized in the ciliate *Tetrahymena thermophila* and found to consist of tandemly repeated arrays of TTGGGG sequences (2). Telomeric sequences (and lengths) vary throughout all organisms, but all consist of a sequential repeats of G-rich sequence. Vertebrate telomeres contain arrays of TTAGGG (3, 4), ranging in length from 5-15 kb in humans to up to 100 kb for some species of mouse (5).

Telomeres and senescence

Replication of linear DNA molecules presents a problem for cells, as the conventional DNA polymerases cannot replicate the extreme 5' termini of chromosomes (the so-called end-replication problem) (Figure 1.1A). Telomeres help to solve this problem, providing a buffer of noncoding DNA and helping maintain chromosomal integrity. Thus, with each round of DNA synthesis, the cell loses a portion of its telomeric DNA. Without some way to combat this telomeric attrition, the telomeres within a cell become progressively shorter over time (Figure 1.1B). Indeed, telomere shortening has been observed over successive cell divisions in human cell lines (6). It

is thought that this progressive shortening of the telomeric DNA leads to a subsequent loss in capping ability, presumably due to disruption of the telomeric chromatin.

In culture, normal human cells will undergo a certain number of divisions before ceasing to divide, the maximal number of divisions achieved is known as the Hayflick limit (7). At this limit, the telomeres have been critically shortened, and the cell enters into at state of proliferative senescence (also known as M1 or mortality stage 1) (see Figure 1.1C and reviewed in [8]). Senescent cells do not divide, but are still metabolically active. A few cells can escape senescence, mostly due to inactivation of cell cycle checkpoint proteins (namely p53 and Rb). They continue dividing (the telomeres becoming ever shorter) until reaching a second proliferative block, termed crisis (or M2, mortality stage 2) (also see Figure 1.1C and reviewed in [8]). Crisis is characterized by severe telomere dysfunction (due to the extreme shortening), including end-to-end fusions and other such genetic abnormalities, and eventual apoptosis. The shortest telomeres (and not bulk telomere length) determine cell survival (9). Very few cells escape crisis, and those that do have found a way to maintain their telomere length.

The evidence outlined above suggests telomeres act as a mitotic clock, recording the number of divisions a cell has undergone and counting down the time until growth arrest. Hence, the residual telomere length predicts the remaining life span of a particular cell. This implies telomere length regulation could have ties to aging in humans, with telomeres growing shorter throughout the life of a particular individual. In support of this, telomeres are significantly longer in cells derived from younger individuals than older ones (6, 10). Further, cell lines obtained from individuals with

premature aging disorders display much shorter telomeres than age-matched controls (11). This data is only correlative, but suggests that an understanding of telomere biology would have important implications for aging research.

Telomeric Structure and Telomere Binding proteins

The majority of the telomeric tract consists of double-stranded DNA, with a long single-stranded overhang at the 3' end. This single-stranded overhang folds back and strand invades the duplex telomeric DNA, forming a structure known as a t-loop (Figure 1.2A) (12). This lariat structure is thought to serve as the protective barrier, masking telomeres from being recognized as broken or damaged DNA by sequestering the very end of the G tail away from the repair machinery. Additionally, the single-stranded G-rich tracts of the telomere can fold form G quartets, higher-order structures formed by Hoogsteen base pairing of G-rich DNA which arise as a byproduct of DNA replication (13). The failure to unwind these G quartets leads to intermittent loss of telomere length (14), so resolution of these structures is required for proper telomere maintenance.

Telomeric DNA is bound by several proteins that function to maintain and regulate the t-loop structure (as well as exerting effects on telomere length homeostasis) (Figure 1.2B and C), collectively these proteins are known as the shelterin complex (reviewed in [15]), but have also been called the telosome (16). Shelterin consists of six subunits, TRF1, TRF2, Tin2, Rap1, TPP1, and POT1, that fractionate as a single complex of approximately 1 MDa in HeLa cells (16). Interference with any of these six subunits (by expression of dominant negative mutants or knockdown of the proteins by RNAi) affects end capping (and telomere length) (17-20), further suggesting

that these proteins exists as one large functional complex to regulate telomere structure in the cell.

TRF1 (telomere repeat binding factor 1) was the first protein determined to bind to mammalian telomeres (21). TRF2 was identified as a paralog to TRF1 (22, 23). Both proteins bind directly along the double-stranded region of the telomere and function to negatively regulate telomere length and promote t-loop formation/stabilization (12, 24-27). Both TRF1 and TRF2 can oligomerize (and in fact each binds the telomere as a dimer), suggesting multiple molecules can bind along an extended telomeric tract (24, 28).

POT1 (protection of telomeres 1) is the only other shelterin component to directly bind to telomeric DNA. Unlike TRF1 and TRF2, POT1 binds to the single-stranded 3' overhang and protects it from degradation by cellular nucleases (29, 30). POT1 also has implications in telomere length regulation. The majority of the experimental evidence suggests POT1 (like TRF1 and TRF2) serves as a negative regulator of telomere length. Expression of a dominant negative form of the protein leads to rapid telomere elongation; similarly, siRNA mediated knockdown of POT1 in human cells also results in extension of telomere length (20, 31). However, other studies point to a positive regulatory role for POT1, as ectopic expression leads to telomere elongation (32).

TPP1 (previously known as TINT1 (33), PTOP (34), and PIP1 [20]) binds to POT1 and enhances its association with single-stranded DNA (35). TPP1 together with Tin2 functions to link together the single-stranded (POT1) and double-stranded (TRF1 and TRF2) telomere binding proteins, thus stabilizing the shelterin complex (19, 34, 36).

Rap1, the final protein of the complex, is recruited to the complex through an interaction with TRF2. Rap1, unlike its yeast homolog, is a positive regulator of telomere length in human cells, as overexpression of this protein leads to telomere elongation (37).

Other proteins have been found associated with mammalian telomeres, but are not part of the core shelterin complex. Nevertheless, these proteins also have important implications for telomere stability. Both tankyrase 1 and 2 have been found associated with TRF1. Tankyrase 1 ADP-ribosylates TRF1, impeding its function by removing it from the telomere and promoting its subsequent degradation (38-41). Tin2 protects TRF1 from this modification, thus stabilizing the interaction of TRF1 with the telomere (18). The MRN complex (consisting of Mre11, Rad50, and Nbs1) is also found associated with telomeres (through an interaction with TRF2) (42). This complex normally serves as a DNA damage sensor and functions in recombinational repair (43). The precise role(s) of this complex at telomeres is not known, but it is presumed to function in t-loop formation (44), as similar steps are involved in both processes (generation of 3' overhangs and strand invasion into homologous duplex DNA). In support of this, ATM and ATR (phosphatidyl-inositol kinase-like protein kinase, PIKK, family members and downstream effectors of the MRN complex) are recruited to telomeres during DNA replication and help promote strand displacement by single-stranded telomeric overhangs (45). Also associated with telomeres are the BLM and WRN helicases (46, 47). These helicases are predicted to help resolve G quadruplexes at the telomere end and could also function in t-loop formation. Both helicases have been shown to interact with POT1 (48), providing an explanation for POT1 serving as a

positive regulator of telomere length (as resolution of G quadruplexes is essential for telomere extension).

Telomerase

Telomeric repeats are replenished by the ribonucleoprotein (RNP) enzyme telomerase. Activation of telomerase balances telomere loss and extends the number of divisions the cell may undergo, allowing cells to divide indefinitely (49). First identified in cell extracts from the ciliate *Tetrahymena thermophila* (50), telomerase is composed of an RNA subunit (telomerase RNA or TR), a catalytic reverse transcriptase (TERT), as well as several associated proteins (Figure 1.3). TR contains a short motif with sequence complementarity to the telomeric repeats (51) that serves as the template for repeat addition (52). The *de novo* addition of telomeric repeats to the 3' ends of the chromosomes is catalyzed by TERT (53, 54). TR and TERT comprise the functional core of telomerase, and are the minimal components for *in vitro* assembly of the enzyme (55).

Telomerase RNA (TR)

TRs vary in different organisms with regards to RNA sequence and secondary structure, as well as requirements for transcription, processing and modification, and assembly into RNP complexes (reviewed in [56, 57]). For example, vertebrate and yeast TRs are transcribed by RNA polymerase II (58-60), whereas ciliate TRs are pol III transcripts (52). Transcription of yeast and human TRs by pol II continues beyond the 3' end of the RNA. In yeast, the 3' end is polyadenylated, TMG (tri-methyl guanosine) capped and subsequently processed to remove the poly (A) tail (58, 61). Yeast TRs

associate with Sm proteins (similar to small nuclear RNAs (snRNAs) involved in mRNA splicing), and this association is important to stabilize the telomerase RNP (61).

Human telomerase RNA (hTR) also undergoes several post-transcriptional modifications, including capping at the 5' end and processing at the 3' end to form the mature, functional RNA (62, 63). Proper processing of the 3' end requires a motif found in the 3' half of the RNA, the H/ACA motif (62, 64) (see Figure 1.3A). The H/ACA motif (like the Sm motif in yeast) is also important for hTR stability (64). This H/ACA motif is common to all vertebrate TRs (59), but was first identified in a class of modification guide RNAs, the H/ACA small nucleolar (sno)RNAs. These H/ACA guide RNAs bind to a set of four core proteins, dyskerin (Cbf5 in yeast), Gar1, Nop10, and Nhp2, and direct the cleavage and pseudouridylation (base isomerization of uridine residues) of ribosomal RNAs (reviewed in [65-67]). hTR also binds these four core proteins through its H/ACA motif and requires these proteins for stability *in vivo* (68-71).

Telomerase Reverse Transcriptase

TERT proteins have been identified in a variety of species from yeast to humans (53, 54), and a high degree of sequence conservation exists among species with regard to the reverse transcriptase motifs and active site of the protein. TERT can be divided into four main regions: the TERT essential N-terminal domain (TEN), the TERT high-affinity RNA-binding domain (TRBD), the central region of the protein (which contains the catalytic core), and C-terminal domain (Figure 1.3B) (56). Comparison of amino acid sequences has shown that the seven characteristic motifs found in viral reverse transcriptases (RTs) are all present in the central region of TERT (54), suggesting a similar catalytic mechanism is employed between viral RTs and telomerase. The other

three regions of the protein are specific for telomerase function. The TEN domain in *Tetrahymena thermophila* can associate with the 3' end of a template telomere (72), linking the enzyme to its substrate. This region in the N terminus contains highly basic residues and a region known as the DAT (dissociates activities of telomerase) domain. In humans, the DAT domain is postulated to be important for telomerase recruitment to the telomere (73). The TRBD domain contains the T motif, a region important for binding of TERT to TR in yeast, humans, and ciliates (74-76). In humans, the C terminal domain is essential for function, as mutations in this domain (as well as adding residues to the C terminus of the protein) abrogate its function *in vivo* (77).

Other proteins of the telomerase RNP

Besides TERT and the H/ACA snoRNP proteins, several other proteins have been found to associate with the telomerase RNP. These include the survival of motor neurons protein (SMN) (78), the Sm B/B' and D proteins (79), the hnRNP proteins A1 (80, 81), C1 and C2 (82), and D (83), the vault protein Tep1 (84), La (85), Stauffen and L22 (86), the chaperones p23 and p90 (87, 88), and hEST1 (89, 90). Each of these proteins was identified separately through various genetic and biochemical approaches. How they enter and exit the telomerase complex to regulate telomerase function remains poorly understood. None of these proteins is specific to the telomerase RNP; each has been shown to interact with another RNA or protein, making study of the specific effects on telomerase difficult.

Telomerase Regulation

Genome stability requires a balance between the loss and gain of telomeric sequences. Given the importance of maintaining this equilibrium, one would think

telomerase would be constitutively active (similar to housekeeping genes). Instead, telomerase is highly regulated in human (and other mammalian) cells. All human somatic cells transcribe and accumulate hTR at appreciable levels; however, the presence of catalytically active telomerase is much more restricted. Telomerase inactivity is most often correlated with an absence in hTERT expression (91), making TERT the limiting component for enzyme activity (92, 93). The regulation of telomerase occurs at various levels, including synthesis and turnover of the core components, assembly into active telomerase complexes, intranuclear trafficking and subcelluar localization of telomerase components, and recruitment of telomerase to telomeres. A number of these are discussed below.

Telomerase and Development

Human telomerase expression is developmentally regulated. Telomerase activity can be found in developing embryonic tissues and cells, but not in the majority of adult somatic tissues (94, 95). Telomerase is detectable at high levels in blastocyst cells and can be seen in fetal tissues throughout the first trimester of pregnancy (95). Telomerase is thought to be active early in development to offset the telomere loss that would occur due to the rapid cycles of proliferation experienced by cells during differentiation. Throughout gestation, telomerase activity is downregulated in a number of tissues, starting first in brain and bone tissues, and remaining longer in the liver, spleen, lung, and testes (96). Following this global downregulation in telomerase expression, the enzyme remains inactive in most tissues throughout the lifespan of an individual. Exceptions to this include highly proliferating cell and tissue types, such as bone marrow stem cells, and germ cells, which maintain active telomerase and

telomere length maintenance (95). Thus, without telomerase to maintain them, telomeres grow gradually shorter over an individual's lifetime (and telomere length corresponds to age, see above).

hTERT regulation

Transcriptional control of hTERT expression is thought to be the predominant method of telomerase regulation in human cells. Indeed, shut down of transcription from the hTERT promoter is the way telomerase expression is downregulated in developing human embryos (96). The hTERT promoter contains no TATA or CAAT boxes, and instead is highly GC rich (suggesting methylation is the primary means of regulation, although studies are conflicting) (97). There are binding sites for multiple transcription factors, indicating that hTERT expression is regulated at many different levels. Positive regulators of hTERT expression include c-Myc (an oncogene which promotes proliferation and has effects on genes involved in cell cycle control) and steroid hormones, while negative regulators (i.e. those that repress telomerase expression) include Mad1 (effects similar genes as c-Myc, functions instead to repress their transcription), pRB, and p53 (both important cell cycle control genes)(reviewed in [97]).

In addition to transcriptional control, hTERT mRNA is differentially spliced and the alternatively spliced products can be detected in human cells (98). Catalytic activity is only detected when the full length (not alternatively spliced) product is expressed. The various spliced transcripts are expressed during different stages of development in a tissue-specific manner (99, 100), but the function of these alternatively spliced products remains unknown.

Together, transcriptional control and alternative splicing of the TERT transcript function to regulate the levels of TERT mRNA available for synthesis into functional TERT protein. TERT is also posttranslationally modified, providing an additional layer of control to regulate TERT function. Accumulating evidence suggests TERT is regulated by phosphorylation, a modification known to regulate protein structure, localization and activity. The phosphorylation of TERT has been shown to affect its subcellular localization; in T lymphocytes phosphorylation of TERT is important for transport of the protein from the cytoplasm to the nucleus (where telomerase ultimately functions) (101). Phosphorylation of TERT has been linked to protein kinase C (PKC), a phospholipid dependent kinase involved in cell growth and differentiation, and the Akt kinase, which is also involved in cell proliferation and survival, as well as the tyrosine kinase c-Abl, which functions to inhibit telomerase function (reviewed in [97]).

hTERT levels are also managed by ubiquitin-mediated degradation of the protein. Ubiquitation targets specific proteins for degradation by the proteosome and is carried out by a cascade of reactions catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) (102). The E3 proteins provide specificity to the reaction, recognizing the protein to be targeted for destruction. MKRN1 (Makorin RING finger protein 1) is an E3 ligase shown to interact with the C-terminus of hTERT and mediate ubiquitination of the hTERT protein (103). Overexpression of MKRN1 in cancer cells leads to hTERT degradation and decreases in telomerase activity, ultimately resulting in telomere shortening (103).

Subcellular localization of telomerase components

Once synthesized, TR and TERT need to be assembled into an active enzyme prior to serving out their functions in telomere length maintenance. Where within the cell this assembly takes place, as well as other steps in the maturation and trafficking of the telomerase RNP remain to be elucidated. To more precisely understand these processes, we and others have determined the intranuclear localization of telomerase components (63, 104-109). The physiological significance of these localization patterns should give insight into how telomerase assembly and function are regulated in the cell.

Initial studies into the subnuclear distribution of telomerase RNA were conducted in the *Xenopus* oocyte system (105, 106). These studies implicated two nuclear structures in the trafficking of TR, Cajal bodies and nucleoli (105, 106). More recently, these structures have also been implicated in the trafficking of telomerase in human cells (as outlined below).

Localization to nucleoli

Localization studies utilizing YFP- and GFP- tagged hTERT proteins have shown that the protein is dispersed throughout the nucleoplasm, but is concentrated primarily in nucleoli in human cell lines (104, 107, 109). The localization of TERT switches between these two compartments in response to cell cycle phase, DNA damage, and oncogenic transformation (109). Further, YFP-hTERT is found in nucleoli of hTR-negative VA13 cells, suggesting that the nucleolar localization of hTERT can occur independently of hTR expression (104). A small fraction (estimated to be less than 10%) of hTR is also in nucleoli by subnuclear fractionation of HeLa cell nuclei (64).

Localization to Cajal bodies

To refine our knowledge of hTR localization, we (and others) developed a fluorescence *in situ* hybridization (FISH) procedure specific for detection of hTR, obtaining the first detailed glimpse of its intranuclear distribution (63, 108). Using this technique we found that hTR localizes to intranuclear Cajal bodies in telomerase-positive cancer cell lines, but not telomerase-negative primary lines (Appendix 1 (108), see Figures A1.2, A1.3, and A1.4). A small fraction of YFP-hTERT also accumulates within this nuclear structure (Appendix 1 (108), see Figure A1.6). Together this data led us to hypothesize that telomerase is assembled in Cajal bodies.

Cajal bodies, sites of RNP (and telomerase) biogenesis

Cajal bodies (CBs) are small spherical subnuclear structures originally described by the Spanish scientist Ramon y Cajal in 1903 (110). CBs vary in size from 0.2 to 2 microns and can be found in the nuclei of plant, insect, and animal cells (reviewed in [110, 111]). The precise function of this nuclear body remains unclear, but the CB is proposed to be a site of biogenesis for several cellular RNPs, including snRNPs (small nuclear ribonucleoproteins, involved in pre-mRNA splicing) and snoRNPs (small nucleolar ribonucleoproteins, involved in ribosome biogenesis) (Figure 1.4 and reviewed in [112]).

A significant portion of snRNP maturation and assembly takes place within the cytoplasm, but additional maturation of snRNPs occurs within CBs. In line with this notion, localization studies using tagged snRNP proteins showed they are transported into the nucleus where they enter into CBs prior to reaching their steady-state localization in speckles (sites of storage for snRNPs) (113). The CB is home to a newly

discovered class of modification guide RNAs, the small Cajal body (sca)RNAs (114-116). These RNAs are known to carry out the posttranscriptional site-specific pseudouridylation and 2'-O-methylation of snRNAs within the CB (117), an important step in modulating the function of these snRNAs.

Both H/ACA and C/D snoRNAs and the core snoRNP proteins are found in CBs, and the CB could be the site for their assembly into an RNP complex (110). Injection studies in *Xenopus* oocytes have shown that snoRNAs transit through CBs before reaching their functional site, the nucleolus (118). The SMN complex (survival of motor neurons protein + 7 gemin subunits), a known RNP chaperone, is found within CBs (119, 120), where it may function to mediate snoRNP biogenesis. SMN is known to interact with the snoRNP proteins fibrillarin (121) and Gar1 (122), supporting a role for this RNP chaperone in snoRNP assembly. Tgs1, the methyltransferase responsible for 5' cap tri-methylation of certain box C/D snoRNAs (important modification for stability), is also present in Cajal bodies(123), and this modification step is predicted to take place within CBs (123).

Based on our preliminary evidence (Appendix 1 [108]) we hypothesize that TR is assembled with TERT into an active telomerase complex within the CB, adding another RNP to the diverse list of those assembled within this nuclear structure. Other assembly and maturation steps for the telomerase RNP can be envisioned to occur within the CB. The 5' end of mature hTR contains a tri-methyl cap and this modification likely takes place in CBs (63). Perhaps assembly of hTR with the H/ACA snoRNP proteins occurs in CBs, as these proteins are present in this nuclear structure ([110] and see above). In addition, telomerase has been shown to physically associate with SMN

(78), directly linking the telomerase RNP with a proposed chaperone (that is present within CBs) for its assembly. Furthermore, hTR may be a target of modification by scaRNAs within the Cajal body, although no such modifications have yet been mapped for hTR.

Telomerase access to the telomere

Once telomerase is assembled from its core components (TR and TERT) it must gain access to the 3' end of telomeres in order to carry out its function (extension of telomeric repeats). Recent data has given insight into this process, increasing our knowledge of the regulation of telomerase and telomere length homeostasis. Elegant *in vivo* analysis of telomere elongation in yeast has shown that not every telomere is extended by telomerase within a given cell cycle, with preference given to the shortest telomeres in a population (124). Telomerase also exhibits a preference for short telomeres in human and mouse cells; when telomerase is limiting in these cells, the shortest telomeres are elongated first (125, 126).

Moreover, telomerase seems to be regulated to function only during S phase of the cell cycle. In humans telomeres are replicated throughout S phase (127, 128), and telomere synthesis is thought to coincide with telomere replication in these cells. Likewise, in yeast, telomerase acts on telomeres in late S phase (and also into G2) (129, 130). Recent data suggest this restriction in timing is due to recruitment of specific proteins to the telomerase RNP and/or subcellular trafficking of telomerase components.

In yeast, chromatin immunoprecipitation (ChIP) assays have revealed that TR and TERT are a stable (but not functioning) component of the telomeric chromatin

throughout the cell cycle. In G1, the telomere binding protein Ku recruits TR to the telomere (131). This recruitment is not essential for telomerase function and is instead thought to enhance association of telomerase with the telomere(s), as Ku mutants display short, but stable, telomere lengths (132). During S phase, the telomerase-associated protein EST1 becomes associated with telomeres, converting the inactive telomerase into an active form (Figure 1.5A) (133). The specific timing of association of EST1 suggests that this recruitment is responsible for restricting telomerase function to late S phase.

In ciliates, access of telomerase to the telomere appears to be regulated at the level of subnuclear trafficking. In nonreplicating cells, TR is found in spherical foci enriched in hypermethylated RNAs (134). Specifically during S phase TR is mobilized from these foci to the replication band, the site of DNA (and telomere) synthesis (134), corresponding to the time when DNA synthesis (and telomere synthesis) takes place (Figure 1.5B). This suggests that telomerase is sequestered away from its substrate (the telomere) at times outside of S, most likely to prevent the enzyme from extending non-telomeric sites (e.g. double-strand breaks).

In humans, the components of the shelterin complex are thought to regulate access of telomerase to telomeres (Figure 1.6). In line with this, TRF1 functions to negatively regulate telomere length. Expression of a dominant negative form of the protein leads to telomere lengthening in telomerase-positive cells, but not telomerase-negative cells (27, 135), suggesting the protein inhibits telomere elongation by telomerase. Longer telomeres bind more TRF1, and thus are probably not as likely to recruit telomerase as a shorter telomere. Thus, TRF1 is thought to provide some sort of

counting mechanism, ensuring that the shortest telomeres are extended first by telomerase (Figure 1.6). TRF2 also functions as a negative regulator of telomere length. Consistent with this role, overexpression of TRF2 leads to telomere shortening (26). TRF2 is essential for t-loop formation; formation of this structure occludes the 3' end of the telomere (12), preventing an association with telomerase.

Recent data has shown one component of shelterin, TPP1, associates directly with telomerase, providing the link between shelterin and telomerase (35, 136). Unlike TRF1 and TRF2, TPP1 has a stimulatory effect on telomerase function, influencing activity and processivity of the enzyme (136). Moreover, TPP1 is thought to recruit telomerase to the telomere (35). TPP1 is thus a critical regulator of telomerase function, although how this regulation is manifested in the cell has yet to be uncovered. Particularly of interest will be whether or not TPP1 can trigger preferential recruitment of telomerase to the shortest telomeres and how TPP1 functions to recruit telomerase only during S phase.

Telomerase and Disease

Besides the obvious role in maintaining genomic stability, the regulation of telomere length and telomerase activity has consequences for human health. As previously discussed, telomere shortening has been linked to human aging. Telomerase also has implications in several human diseases; misregulation of telomerase activity (and telomere length maintenance) can lead to these disease states. Overabundance of telomerase has links to human cancer and deficiency has implications for a disease known as dyskeratosis congenita. Both are discussed below.

Telomerase and Cancer

The vast majority of human cancer cell lines and tumors possess active telomerase, maintaining stable telomere lengths and thereby enabling continued proliferation (Figure 1.7) (94, 137). Telomerase activity (as well as detection of hTERT) has been used as a biomarker for cancer screening and diagnosis (reviewed in [138]). Knockdown of telomerase activity in these cancer cells (by targeting either the RNA or TERT component) leads to telomere shortening and eventual cell death (reviewed in [139]). The telomerase RNP is thus an attractive target for the design of novel anti-cancer strategies. The majority of human somatic tissues do not contain telomerase activity (see above) and should be largely unaffected by these anti-telomerase treatments. An understanding of telomerase regulation has important implications for the design of such treatments. The challenge lies in exploiting the information known about telomerase assembly and recruitment to telomeres in order to intervene in these processes in cancer cells.

Dyskeratosis congenita

Dyskeratosis congenita (DC) is a rare inherited bone marrow syndrome that is characterized by a number of symptoms, including abnormal skin pigmentation, nail dystrophy, leukoplakia (a condition of the mouth characterized by white leathery spots on the tongue and inside the mouth), and bone marrow failure (which ultimately leads to death of the affected individual) (140). The disease is classified into three main forms, based on its inheritance pattern (reviewed in [141]). X-linked (recessive) DC is the most common form, and is due to mutations that encode the gene dyskerin (an H/ACA snoRNP protein that binds TR). The mutations associated with this form of the disease

result in an hTR accumulation defect (70). A second subtype of DC is inherited in an autosomal dominant form. Patients with this form of the disease are heterozygous for the allele encoding TR, thus disease states result from a haploinsufficiency of hTR (i.e. one wild-type copy of the gene is not sufficient to maintain hTR levels). Some forms of autosomal dominant DC arise due to mutations in hTERT, the catalytic subunit of telomerase (142) (which also leads to a haploinsufficiency of telomerase). The final form of DC is autosomal recessive and has recently been linked to mutations in Nop10, another of the H/ACA proteins (143).

DC is classified as a telomere/telomerase disorder, as the two best studied forms of the disease are known to effect components of the telomerase complex (141) (and at least one subtype of the third form is connected to a telomerase-associated protein—Nop10 [143]). Indeed, telomeres from DC patients are shorter than age matched controls (144, 145). This is most likely due to lower levels of telomerase in these cells (due to reduced levels of hTR). Also, the mutations in TR exhibited by autosomal dominant DC lead to reduced association of TR with TERT in *in vitro* assays (146). Most likely this reduced ability to associate with TERT is due to changes in the secondary and tertiary structure adopted by the mutant TR (147). Lastly, the symptoms of DC develop predominantly in tissues with high proliferation rates and/or cellular renewal (e.g. the skin and bone marrow), somatic tissues that do have detectable levels of telomerase activity.

Overview

Regulation of telomerase function has important implications in genome maintenance as well as human disease. In order to carry out its biological function,

telomerase must be assembled and then transported from that site of biogenesis to its functional destination at telomeres. Knowledge of how these steps are regulated and where within the cell these processes occur would have a tremendous impact on our understanding of basic telomerase biology. With that in mind, we have begun exploring the subnuclear distribution of telomerase components.

As described above, we developed a FISH procedure specific for the detection of human telomerase RNA, and were the first to describe its intranuclear distribution (Appendix 1 [108]). Using this technique, we discovered that hTR localizes to Cajal bodies in telomerase-positive human cancer cells, but not in telomerase-negative normal cell lines. This finding was later substantiated by the Kiss laboratory, who went on to find an S phase enrichment of hTR within CBs in the cancer lines (63).

In Chapter 2 we describe a dynamic, S phase-specific trafficking of the telomerase RNP. By independently examining the localization of hTR and hTERT across the cell cycle, we show that these components are housed in separate nuclear foci throughout most of interphase; hTR within Cajal bodies (as detailed above and see Appendix 1 [63, 108]) and hTERT in as yet unidentified foci. During S phase, the components are mobilized, first to nucleoli, then to Cajal body-associated foci and to specific subsets of telomeres. This work was significant, as it marked the first time telomerase was viewed at the telomere.

In Chapter 3 we set about to better understand this trafficking pathway, and examine factors that may influence the localization of hTR. We found that expression of hTERT is essential for localization of hTR to both Cajal bodies and telomeres. Further,

expression of hTR enhances the localization of hTERT to both telomeres and Cajal bodies, indicating trafficking and biogenesis are closely linked.

Chapter 4 describes development of a FISH procedure to detect telomerase RNA (mTR) in cultured mouse cell lines. Using this technique, we discovered that mTR does not localize to Cajal bodies (in the majority of cell lines examined) and instead resides in separate nuclear foci. Similar to human telomerase, mTR is associated with specific subsets of telomeres during S phase of the cell cycle. Hopefully, we will be able to exploit the power of mouse genetics and utilize this system to study other factors that mediate the trafficking of telomerase to telomeres.

In Appendix 2 we examine the early steps in the trafficking of TR using the *Xenopus* oocyte system. TR injected into the nuclei of these oocytes is rapidly assembled with the endogenous TERT to form an active RNP. The timing of this assembly coincides with localization of TR to CBs, and suggests that assembly could be occurring within this nuclear structure. Further, we find that enzyme assembly is more than likely not occurring within nucleoli, as assembly precedes localization of TR to nucleoli. Also, mutations in the H/ACA motif (which prevent association of TR with nucleoli, see [105]) do not prevent telomerase assembly *in vivo*.

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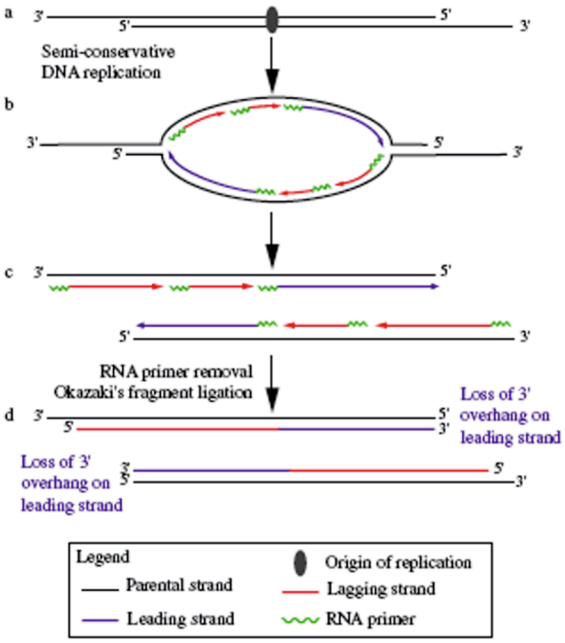
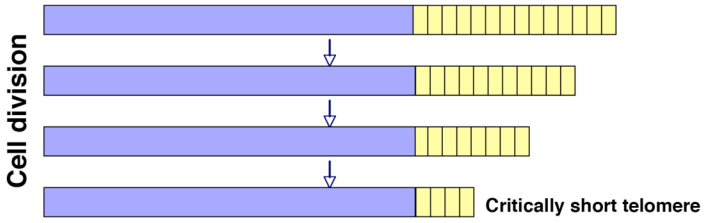
Figure 1.1. The gradual shortening of telomeres caused by the end replication problem leads to cellular senescence and eventual cell death. *A. The end replication problem.* The mechanism of semi-conservative DNA replication is shown (adapted from [148]). For simplicity, only one origin of replication (grey oval in [a]) is depicted, although multiple origins are firing simultaneously along the chromosome. Replication forks move in opposite directions from the origin. Because DNA polymerases are unidirectional (synthesis in the 5' to 3' direction), each replication fork contains a leading and lagging strand (b). Synthesis along the leading strand is continuous (synthesis is primed from one RNA oligo), while it is discontinuous along the lagging strand (several RNA primers are employed, generating small stretches of DNA called Okasaki fragments)(c). Sequence is lost on the leading strand, corresponding to the DNA segment encompassed by the RNA primer (d). No net sequence loss occurs on the lagging strand. *B. Telomeres gradually shorten over progressive cell divisions.* Depicted is one chromosome over several cell divisions. Yellow boxes indicate tracts of telomeric repeats. Due to the end replication problem (outlined in A), a portion of the telomeric DNA is lost with each round of DNA synthesis in progressive cell division cycles. Eventually, the telomere(s) reaches a critically short length. *C. Telomere shortening is linked to senescence and crisis.* Figure from (8). As primary cells in culture continue to divide, the telomeres gradually shorten (as indicated in B). Eventually the telomeres reach a critically short length and cause the cell to enter into a state of proliferative senescence (M1). Senescence can be bypassed if cell cycle control genes (such as Rb and p53) are lost. The cell continues to divide and the telomeres become even shorter until the cell eventually reaches M2, or crisis. This

stage is characterized by multinucleate cells (darker grey ovals) and eventual cell death. A few cells escape crisis, they become immortal upon sustained telomere maintenance.

A


a
3'
5'
5'
3'
Semi-conservative
DNA replication
b
3'
5'
5'
3'
c
3'
5'
5'
3'
RNA primer removal
Okazaki's fragment ligation
d
3'
5'
5'
3'
Loss of 3'
overhang on
leading strand
Loss of 3'
overhang on
leading strand
5'
3'
Legend
Parental strand
Leading strand
Origin of replication
Lagging strand
RNA primer

B


Cell division
Critically short telomere

C

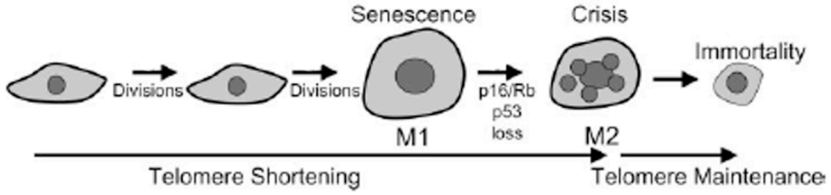
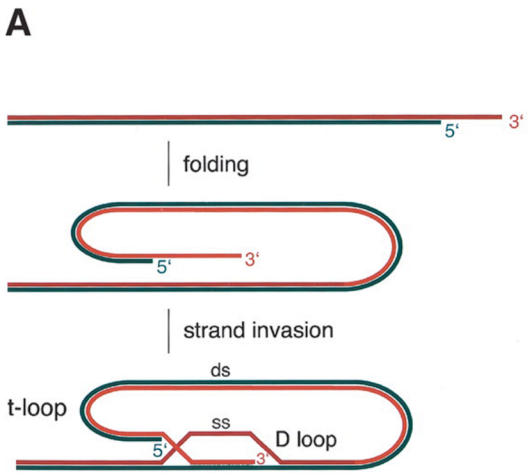
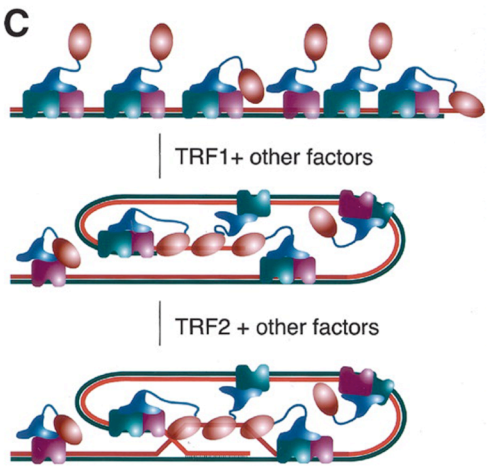

Senescence
Crisis
Immortality
Divisions
Divisions
p16/Rb
p53
loss
M1
M2
Telomere Shortening
Telomere Maintenance

Figure 1.2. Shelterin components bind the telomere and may assist in t-loop formation. *A. Telomeres adopt a t-loop conformation.* Telomeres consist of long tracts of duplex DNA and a single-stranded 3' overhang. T-loops are formed as the duplex DNA bends back on itself, positioning the single-stranded (ss) overhang to strand invade into the duplex telomeric DNA (ds). The strand invasion results in displacement of a strand of the duplex, resulting in a D loop (displacement loop). *B. The shelterin complex binds to telomeric DNA.* The shelterin complex consists of six subunits: TRF1, TRF2, Rap1, TIN2, TPP1, and POT1. TRF1 and TRF2 bind to the double-stranded region of the telomere, often as dimers (as indicated). Rap1 is recruited to the telomere through an interaction with TRF2. POT1 binds directly to the single-stranded telomeric overhang. TIN2 and TPP1 do not bind the telomere directly, and instead bridge the gap between the double-stranded telomere binding proteins (TRF1, TRF2, Rap1) and POT1. *C. Shelterin components may assist in t-loop formation.* Shelterin components bind along the telomere in repetitive tracts. TRF1 (and other factors) have been shown to bend duplex DNA, and binding of this protein may facilitate the folding of telomeric DNA, thus positioning the 3' end for strand invasion. TRF2 is essential for t-loop formation (although the role it plays is largely unknown). Figure adapted from (15).


A
5'
3'
folding
5'
3'
strand invasion
ds
t-loop
ss
D loop
5'
3'


C
TRF1+ other factors
TRF2 + other factors

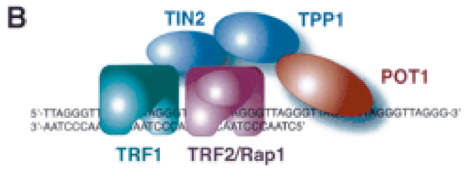
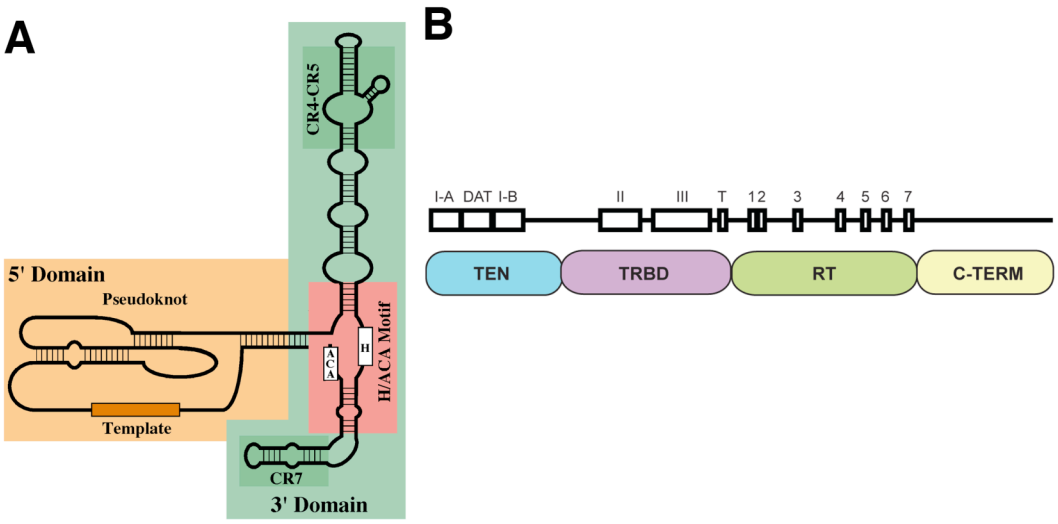

B
TIN2
TPP1
POT1
TRF1
TRF2/Rap1

Figure 1.3. Telomerase is an RNP enzyme. *A. Predicted secondary structure of human telomerase RNA.* Analysis of the sequences of over 30 different vertebrate TRs has resulted in a consensus secondary structure (59). The template region is found in the 5' end of the molecule, while the H/ACA motif is found in the 3' end. The 5' pseudoknot and 3' CR4/5 domain are important for TERT interaction. *B. hTERT is comprised of several domains.* Shown is a schematic of the 1132 amino acid hTERT protein. The TERT essential N-terminal domain (TEN) contains the DAT (dissociates activities of telomerase) domain and is important in telomere recruitment of the enzyme. The TERT high-affinity RNA-binding domain (TRBD) contains the regions essential for binding to telomerase RNA, including the T motif. The catalytic core is found in the central region of the protein (RT, reverse transcriptase). Comparison of amino acid sequence has shown that hTERT contains the seven characteristic motifs found in viral reverse transcriptases (1-7). The C terminal portion of the protein (C-TERM) is also important for *in vivo* function of telomerase. Figure adapted from (56). *C. The telomerase RNP.* The 451 nucleotide telomerase RNA is bound by TERT, as well as the H/ACA snoRNP proteins dyskerin, Gar1, Nop10, and Nhp2 (through the H/ACA motif). Presumably other proteins associate with the telomerase RNP to regulate its function. Figure adapted from (141).


A
CR4-CR5
5' Domain
Pseudoknot
H/ACA Motif
H
ACA
Template
CR7
3' Domain
B
I-A DAT I-B
II
III
T
12
3
4
5
6
7
TEN
TRBD
RT
C-TERM

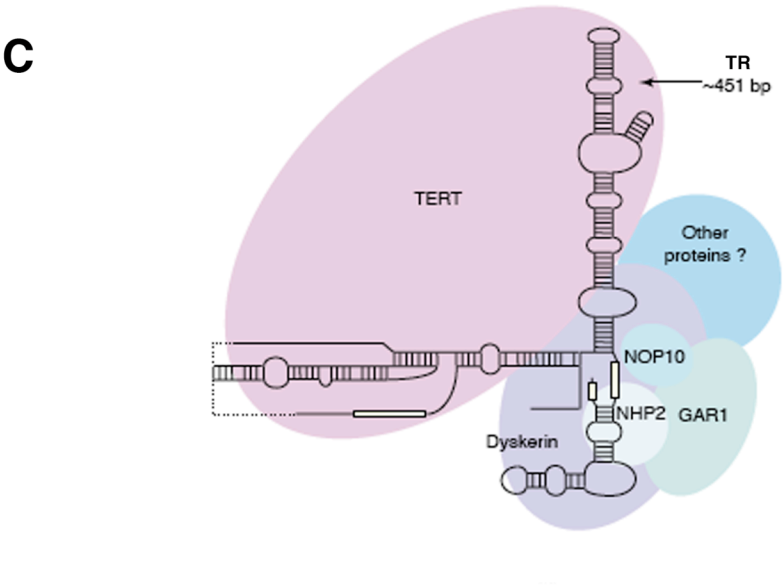

C
TR
~451 bp
TERT
Other
proteins ?
NOP10
NHP2
GAR1
Dyskerin

Figure 1.4. Cajal bodies are presumed sites of RNP maturation and assembly. Cajal bodies (CBs) are enriched in components of the snRNP (small nuclear RNP, red pathway) and snoRNP (small nucleolar RNP, blue pathway) maturation pathways. Following RNP assembly (in the cytoplasm), snRNAs are site-specifically pseudouridylated or 2'-O-methylated by scaRNAs (small Cajal body RNAs) within the CB before proceeding to their sites of storage, the speckles. Both H/ACA and C/D snoRNAs as well as their associated proteins traverse CBs prior to arrival at their functional site, the nucleolus. The CB is predicted as the site of snoRNP maturation and assembly (including 5' cap hypermethylation of a subset of C/D snoRNAs). The Cajal body is also home to the SMN (survival of motor neurons) complex, a known RNP chaperone.

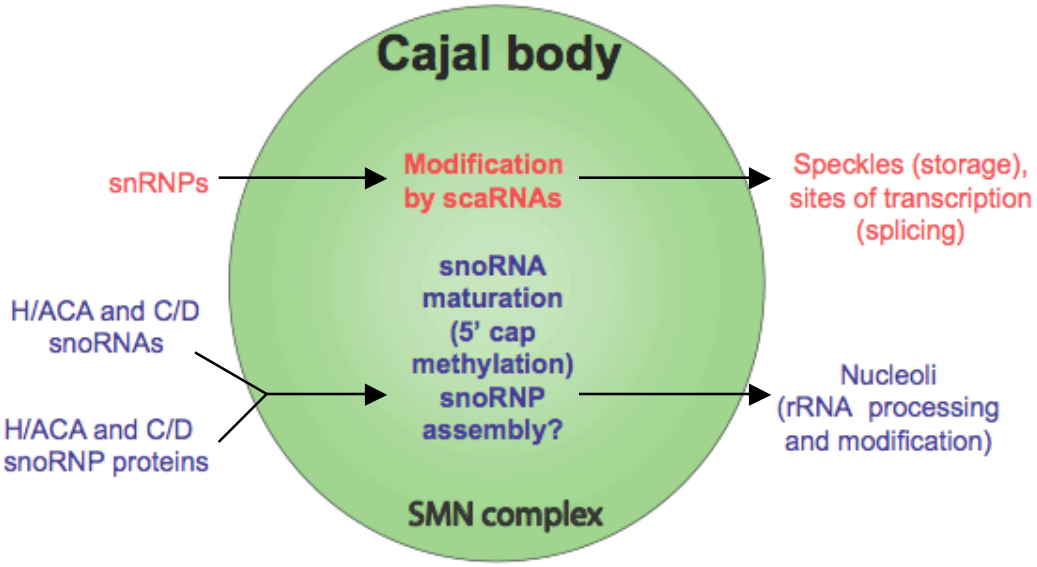
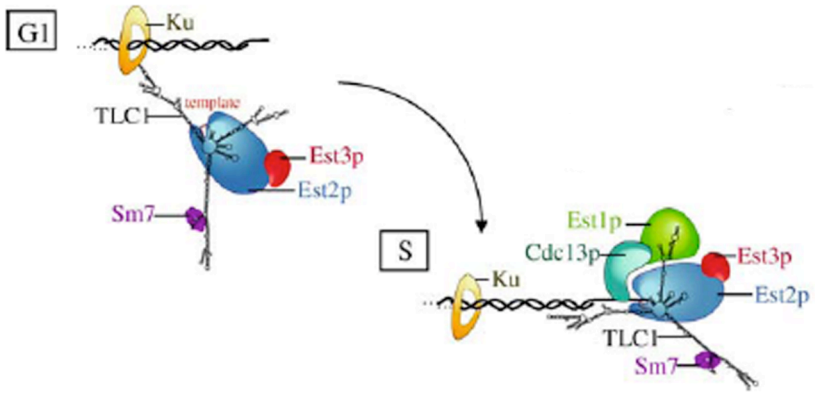

Cajal body
snRNPs
Modification by scaRNAs
Speckles (storage), sites of transcription (splicing)
snoRNA maturation (5' cap methylation) snoRNP assembly?
H/ACA and C/D snoRNAs
H/ACA and C/D snoRNP proteins
Nucleoli (rRNA processing and modification)
SMN complex

Figure 1.5. Regulation of telomerase function during S phase. *A. In yeast, the telomerase complex is remodeled during S phase.* Ku recruits telomerase to the telomere during G1 through an interaction with TR. During S phase, Est1p associates with telomerase (and the single-stranded telomere binding protein Cdc13p), activating telomerase to function in telomere length extension. Figure adapted from (148). *B. In ciliates, telomerase is recruited to telomeres during S phase.* Throughout most of the cell cycle, TR is found in spherical structures (A, indicated by black arrows). During S phase, a small fraction of TR is mobilized to the replication band (the site of telomere synthesis in these cells (B, indicated by white arrowhead). Data taken from (134).

A


G1
Ku
template
TLC1
Est3p
Est2p
Sm7
S
Est1p
Cdc13p
Est3p
Ku
Est2p
TLC1
Sm7

B

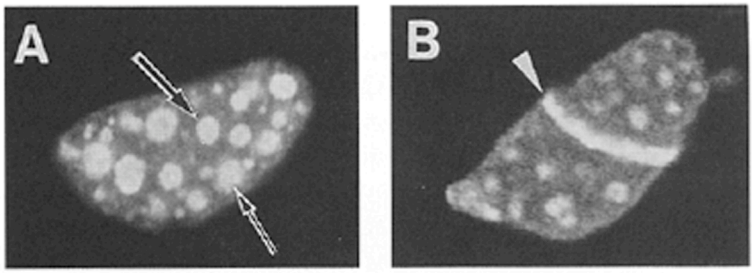

A
B

Figure 1.6. Model of how shelterin regulates access of telomerase to telomeres. Longer telomeres are predicted to recruit more shelterin complexes, ensuring proper t-loop formation. This, in turn, is predicted to block telomerase from extending the 3' end. In contrast, shorter telomeres recruit fewer shelterin complexes and cannot efficiently block telomerase from the end. Figure adapted from (15).

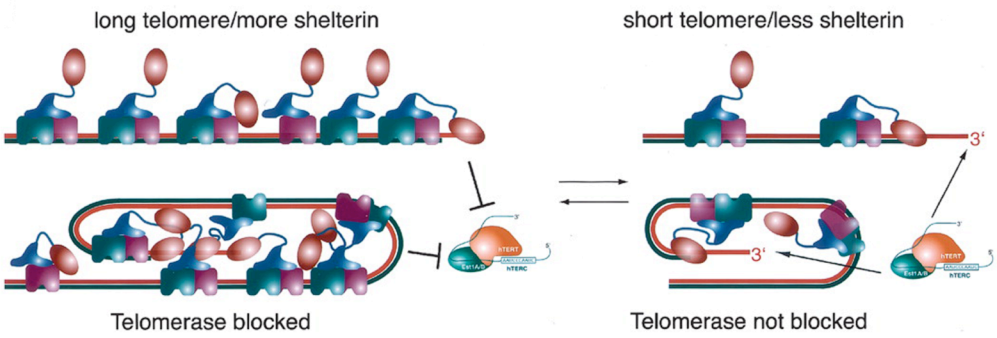
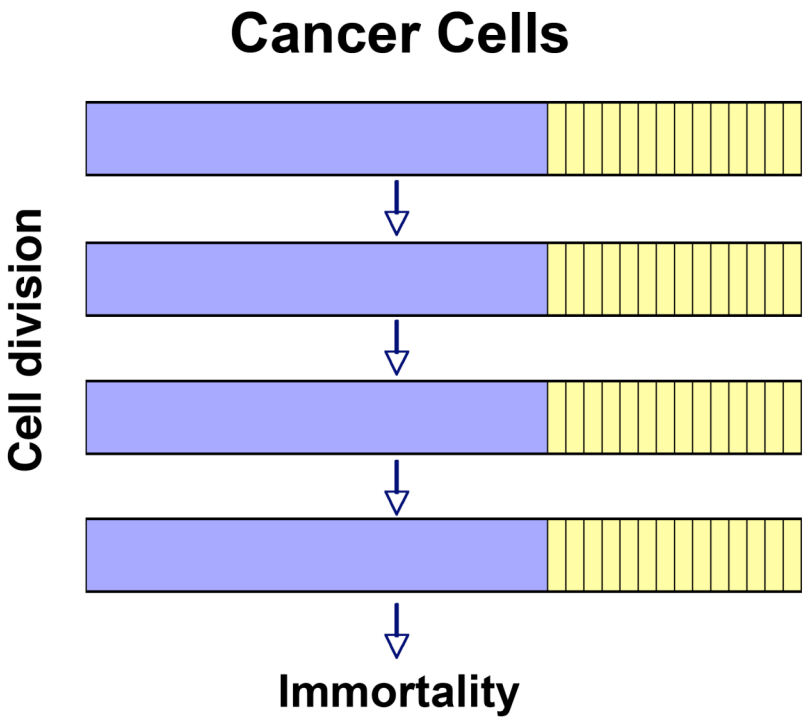

long telomere/more shelterin
short telomere/less shelterin
3'
3'
hTERT
hTERC
Telomerase blocked
Telomerase not blocked

Figure 1.7. Telomere length is maintained in the majority of human cancer cell lines. Over 90% of human cancers express telomerase, and thus are able to maintain their telomere length over progressive cell divisions (unlike normal cells, see Figure 1.1B). This continued maintenance results in cellular immortality. Depicted is one chromosome in a cancer cell (with active telomerase). Yellow boxes indicate tracts of telomeric repeats which do not shorten with each cell division cycle.


Cancer Cells
Cell division
Immortality

Chapter 2

Cell Cycle-regulated Trafficking of Human Telomerase to Telomeres[1]

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[1]Rebecca L. Tomlinson, Tania D. Ziegler, Teerawit Supakorndej, Rebecca M. Terns, and Michael P. Terns. 2006. *Molecular Biology of the Cell*, Vol. 17, Issue 2, 955-965. Reprinted with permission of publisher.

Abstract

Telomerase synthesizes telomeres at the ends of human chromosomes during S phase. The results presented here suggest that telomerase activity may be regulated by intranuclear trafficking of the key components of the enzyme in human cells. We examined the subcellular localization of endogenous human telomerase RNA (hTR) and telomerase reverse transcriptase (hTERT) in HeLa cervical carcinoma cells. Throughout most of the cell cycle, we found that the two essential components of telomerase accumulate at intranuclear sites separate from telomeres. However, during S phase, both hTR and hTERT are specifically recruited to subsets of telomeres. The localization of telomerase to telomeres is dynamic, peaking at mid-S phase. We also found complex associations of both hTR and hTERT with nucleoli and Cajal bodies during S phase, implicating both structures in the biogenesis and trafficking of telomerase. Our results mark the first observation of human telomerase at telomeres and provide a mechanism for the cell cycle-dependent regulation of telomere synthesis in human cells.

Introduction

The ends of linear eukaryotic chromosomes are capped by nucleoprotein structures termed telomeres. In vertebrates, telomeres consist of simple DNA repeats of TTAGGG bound by several proteins (1, 2). Telomeres serve to maintain chromosome integrity, preventing illegitimate recombination and end-to-end joining (3-5). However, because of the unidirectional nature of DNA polymerases and DNA processing events, some telomere sequence is lost with each round of DNA replication. If this loss is not

compensated, the telomeres will reach a critically short length, triggering the cell to enter a state of replicative senescence or apoptosis (4, 6).

Telomerase is the ribonucleoprotein (RNP) enzyme that synthesizes telomeres. The telomerase reverse transcriptase (hTERT) catalyzes de novo repeat addition using a short motif within the integral telomerase RNA (hTR) as a template (7). These two components are essential for the activity of the enzyme. Human telomere synthesis occurs early in development (8, 9). The majority of adult somatic cells do not have appreciable telomerase activity and telomeres gradually shorten, limiting cell division capacity (10). In the majority of human cancers, however, telomerase is reactivated and provides the sustained proliferative capacity of these cells (11). An understanding of telomerase biology thus has important implications for both cancer and aging.

Telomeres are synthesized during S phase in human cells (12, 13); however, it is unclear how telomerase is restricted to function specifically during this stage of the cell cycle. Some existing evidence is consistent with the idea that the cell cycle-dependent regulation of telomerase could occur at the level of subcellular trafficking. Redistribution of components of telomerase has been observed during S phase, when telomere synthesis occurs. Wong et al showed that the subnuclear distribution of green fluorescent protein (GFP-hTERT) fusion proteins changed from predominantly nucleolar to nucleoplasmic as cells progressed through S phase (14). In similar studies, Yang et al. also reported movement of GFP-hTERT protein during S phase, in this case into nucleoli (15). Regarding the other essential component of the enzyme, Jady et al reported a possible influx of telomerase RNA through Cajal bodies during S phase (16). However, although there is some evidence that both hTR and hTERT move during S

phase, movement to telomeres, and thus a direct link to regulation of function, has not been found in vertebrate cells. More persuasive evidence is available from ciliates, where telomerase RNA localizes to discrete nuclear foci throughout most of the cell cycle, and a fraction of the RNA is mobilized to the replication band, the site of DNA (and telomere) synthesis, during S phase (17).

In this work, we have investigated the subnuclear distribution of endogenous human TR and TERT over the course of the cell cycle and found compelling evidence that regulation of telomerase activity occurs via trafficking of hTR and hTERT in human cells. Our results indicate that hTR and hTERT move to telomeres from separate sites specifically during S phase. hTR is found in Cajal bodies, as we and others have reported previously (16, 18), throughout most of the cell cycle. Here, we provide the first clear evidence that hTERT resides in subnuclear foci that do not correspond to nucleoli, Cajal bodies, or telomeres during most of the cell cycle. The movement of hTR and hTERT to telomeres during S phase is preceded and accompanied by other changes in localization that may relate to biogenesis and/or transport of the components. Our results suggest that hTERT moves to nucleoli and that Cajal bodies containing hTR accumulate at the periphery of nucleoli early in S phase. In addition, we find that both hTR and hTERT localize to foci adjacent to Cajal bodies during S phase, marking a potential site outside of telomeres where both endogenous telomerase components are detected. The implications of our findings with regard to telomerase biogenesis and telomere length regulation are discussed.

Materials and Methods

Cell Culture and Synchronization

Monolayer HeLa cells were grown on coverslips in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (American Type Culture Collection, Manassas, VA) at 37°C with 5% CO2. IMR90 primary fibroblasts were grown in minimum essential Eagle's media (American Type Culture Collection) with 10% FCS. IMR90-hTERT cells (19) were grown in a 4:1 mixture of DMEM and medium 199 (Invitrogen, Carlsbad, CA). Synchronous populations of HeLa cells were obtained by double thymidine block. Briefly, cells were treated with 2 mM thymidine (Sigma-Aldrich) for 18 h, released in normal media for 9 h, and retreated with 2 mM thymidine for another 18 h. Alternatively, HeLa cells were synchronized by treatment with 5 mM hydroxyurea for 24 h after 24 h of serum withdrawal. Cells were fixed at various time points after release and analyzed as described below.

5-Bromodeoxyuridine (BrdU) Labeling

Before fixation, cells were incubated with 100 μM BrdU (Sigma-Aldrich) for 30 min at 37°C. Cells were then rinsed once with 1x phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM Na2HPO4, 2.7 mM KCl, and 1.4 mM KH2PO4, pH 7.4) and fixed with 4% formaldehyde (Electron Microscope Sciences, Fort Washington, PA), 10% acetic acid, and 1x PBS for 10 min at room temperature. After two PBS washes, cells were permeablized in 70% ethanol overnight at 4°C. Cells were denatured in 70% formamide (Sigma-Aldrich), 2x SSC for 5 min at 80°C. After three PBS washes, BrdU was detected using fluorescein isothiocyanate-conjugated anti-BrdU monoclonal antibody (mAb) (BD Biosciences, San Jose, CA; 20 μl of antibody was diluted in 70 μl of 0.05% Tween 20 in

PBS [PBST]) for 2 h at room temperature. After three PBS washes, coverslips were mounted in 90% glycerol, 1 mg/ml p-phenylenediamine, 1x PBS, and 0.1 µg/ml 4',6-diamidino-2-phenylindole (DAPI). If fluorescence *in situ* hybridization (FISH) was to be performed, cells were fixed again in 4% formaldehyde in 1x PBS for 10 min at room temperature and washed twice with PBS.

hTR and Telomere FISH

Probes complementary to different regions of telomerase RNA (nucleotides indicated) or telomere repeats were as follows: hTR 128-183 (probe 1), GCT*GACATTTTT*TGTTTGCTCT*AGAATGAACGGT*GGAAGGCGGCAGGCCGAGGCT*T; hTR 331-383 (probe 2), CT*CCGTTCCTCTTCCT*GCGGCCTGAAAGGCCT*GAACCTCGCCCT*CGCCCCCGAGT*G; hTR 393-449 (probe 3), AT*GTGTGAGCCGAGT*CCTGGGTGCACGT*CCCACAGCTCAGGGAAT*CGCGCCGCGCT*C; and telomere repeats (probe 4), CT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*A. T* indicates aminoallyl-modified thymidines. All probes were synthesized by QIAGEN (Valencia, CA). Probes were conjugated with either Cy3 or Cy5 monofunctional reactive dye according to manufacturer's protocol (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). FISH was performed essentially as described previously ([18], http://www.singerlab.org/protocols) with the following modifications. For each coverslip, 20-30 ng of each Cy3-labeled hTR probe (1-3 above) and 5 ng of Cy5-labeled telomere probe (4) were hybridized overnight at 37°C. After hybridization, cells

were washed twice with 2x SSC, 50% formamide for 1 h at 37°C. Coverslips were mounted as described above.

Coilin and TRF1 Immunofluorescence (IF)

After FISH, cells were washed twice with PBS. Cells were incubated with one or both of the following antibodies at the indicated dilution for 1 h at room temperature: mouse anti-p80 coilin (1:10,000; gift from G. Matera, Case Western Reserve University, Cleveland, OH) and rabbit anti-TRF1 (1:350; gift from Dominique Broccoli [Fox Chase Cancer Center, Philadelphia, PA] and Susan Smith [Skirball Institute, New York, NY]). Cells were washed three times with PBS and then incubated with secondary antibody (1:100 Cy2-conjugated goat anti-rabbit IgG [H+L], 1:100 AMCA-conjugated goat anti-mouse IgG [H+L], or 1:100 Cy5-conjugated goat anti-mouse IgGγ) for 1 h at room temperature. All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). After three PBS washes, slides were mounted as described above. All antibodies were diluted in PBST.

hTERT Immunofluorescence

hTERT IF was performed essentially as described in (20). Cells were washed in PBS and then fixed with chilled acetone for 5 min. After two PBS washes, cells were treated with 2 M HCl at room temperature for 20 min followed by a PBS wash and neutralization with 0.1 M boric acid, pH 8.5, for 10 min. After two additional PBS washes, cells were blocked with 1% bovine serum albumin (BSA) in PBS at 4°C overnight. Cells were incubated with mouse anti-hTERT 2C4 (Abcam, Cambridge, MA; 1:2000-1:5000 in 1% BSA) for 2 h at room temperature, washed three times with PBS, and incubated with Cy2-conjugated goat anti-mouse IgM (Jackson ImmunoResearch Laboratories; 1:100 in

PBST) for 2 h at room temperature. After three PBS washes, the cells were mounted as described above. When hTERT staining was combined with TRF1 or coilin, the hTERT IF protocol was followed using the above-described antibodies at the indicated dilutions except that cy3-conjugated goat anti-rabbit IgG (H+L) was used to recognize TRF1 and mouse anti-coilin was recognized with Cy5-conjugated goat anti-mouse IgGγ antibody. Both secondary antibodies were obtained from Jackson ImmunoResearch Laboratories.

Microscopy

Analysis was performed on a Zeiss Axioskop 2 Mot Plus fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were acquired at 63x or 100x magnification (Plan Apochromat objectives, numerical aperture 1.4) using a cooled charge-coupled device Retiga Exi Fast 1394 camera (Qimaging, Burnaby, British Columbia, Canada) and IPLab Spectrum software.

Results

Novel Patterns of Telomerase RNA Localization during S Phase

Our laboratory developed a FISH procedure specific for the detection of endogenous hTR in human cells and was the first to describe its intranuclear localization (18). Several controls established the specificity of our FISH procedure for hTR (18). Using this technique, we found that hTR localized to Cajal bodies in telomerase-positive human cancer cell lines (18). In the course of that study (performed with asynchronous populations of cells), we noticed that a small percentage of cells displayed different patterns of hTR localization. For example, we observed additional

hTR foci that did not stain with antibodies against coilin (the marker protein of Cajal bodies).

To investigate whether the secondary hTR localization patterns observed at low frequency in asynchronous populations reflected cells in particular stages of the cell cycle, we performed hTR FISH and coilin IF on cell cycle-synchronized HeLa cervical carcinoma cells. HeLa cells were synchronized with a double thymidine block (or hydroxyurea; our unpublished data) and monitored for synchronization efficiency at the indicated time points by BrdU analysis (to distinguish S-phase cells) and DAPI staining (to distinguish mitotic cells). (Fluorescence-activated cell sorting analysis with propidium iodide-stained cells (to examine DNA content) was also performed to confirm the synchronization efficiency in some cases.) The BrdU staining patterns also allowed us to assess the S subphase (i.e., early, mid, and late) status of populations of cells as well as individual cells (21).

As expected, throughout the majority of the cell cycle, hTR localizes exclusively to Cajal bodies (Figure 2.1, G1 and G2). However, during S phase, a dynamic change in the subcellular distribution of hTR was observed in a significant number of cells. hTR remained associated with Cajal bodies in most cells; however, several novel hTR localization patterns were observed, which peaked at distinct points in S phase. Beginning in G1/S and peaking in early S, hTR foci were found in ring-like patterns within the nucleus (Figure 2.1, row 2). In most cells, coilin colocalized with hTR in the rings, as shown (Figure 2.1, row 2). By mid-S phase, the distribution of hTR foci in rings had declined, and hTR occurred in small, nucleoplasmic foci that did not stain with anti-coilin antibodies (Figure 2.1, row 3, denoted by arrowheads). In addition, hTR was

sometimes observed in foci immediately adjacent to Cajal bodies (detailed below). These patterns were specific to S phase; they were not observed in cells in G1 or G2 phases of the cell cycle.

During mitosis, previous research has shown that Cajal bodies dissociate, and then reform during early G1 of the cell cycle (22). We found that hTR localization parallels that of the Cajal body marker protein coilin during cell division, with only a slight temporal delay in reassociation after mitosis. Like coilin, hTR displayed a diffuse localization pattern throughout mitosis (Figure 2.1, row 5 shows hTR diffusely localized in interchromatin region). No association with discrete foci was observed. After mitosis, Cajal body formation precedes hTR accumulation in Cajal bodies. In early G1, Cajal bodies had reformed in nearly all (~90%) of cells examined, but no hTR foci were found in most (~65%) cells (Figure 2.1, row 6). By mid-G1 (15 h postrelease), hTR had reaccumulated in all Cajal bodies (Figure 2.1, row 1). Together, these results demonstrate that the cell cycle has dramatic effects on the localization of hTR and that there is a dynamic, S phase-specific rearrangement of hTR in human cancer cell nuclei.

Telomerase RNA Localizes to the Periphery of Nucleoli during Early S Phase

We found that the ring-like pattern of hTR (and coilin) foci observed during early S phase seems to correspond to localization to the periphery of nucleoli (Figure 2.2A). In addition to the ring pattern, we also observed hTR foci that seemed to be distributed across the surface of a nucleolus in some focal planes (Figure 2.2A). At early S phase, ~17% of cells (370 cells analyzed from 3 separate experiments) contained hTR foci localized around the periphery of a nucleolus. In the majority of cases, the hTR ring was observed around only one nucleolus within a given cell, suggesting a previously

undescribed heterogeneity among individual nucleoli. We did not find telomeres at these rings; telomeres colocalized with hTR/coilin rings in <1% of 1197 cells examined in two experiments. The peripheral nucleolar hTR pattern was less frequent at G1/S and mid-S than at early S phase and was not observed in cells outside S phase.

As shown in Figure 2.1, coilin was found with hTR in the nucleolar rings in the majority (~80%) of cells. The localization of Cajal bodies to the periphery of nucleoli is well documented (indeed, Cajal bodies were initially termed nucleolar accessory bodies; [23, 24]), although an S-phase-specific association has not been described previously. We cannot exclude the possibility that the Cajal bodies and hTR are internal to nucleoli based on the studies presented here, but the distribution of the foci is suggestive of localization on the surface of the nucleolus. Elegant studies in HeLa cells have demonstrated the movement of Cajal bodies to and from nucleoli (25). Our results suggest that Cajal bodies containing hTR move to the periphery of nucleoli during early S phase in HeLa cells.

Telomerase RNA Localizes to Subsets of Telomeres during Mid-S Phase

To ascertain whether the coilin-negative, S-phase-specific hTR foci described above (Figure 2.1, row 3) were at telomeres, we coanalyzed hTR and telomeres in S-phase cells. We performed hTR FISH in conjunction with either telomere FISH (probe directed against telomere repeat sequences) or immunofluorescence with antibodies against the double-stranded telomere binding protein TRF1 (Figure 2.2B). Cells were examined 0 (G1/S), 2 (early S), 4 (mid-S), 6 (late S), and 8 (S/G2) h postrelease from a double thymidine block. At G1/S and early S, we found that hTR colocalized with a few telomeres (1-2/cell) in ~3 and 9% of cells, respectively (562 and 370 cells analyzed from

6 and 3 separate experiments, respectively). During mid-S, there was an increase in both the number of cells that had hTR-telomere associations and in the number of associations per cell. In ~19% of mid-S-phase cells (698 cells from 5 experiments), hTR was found at telomeres—typically one to five per cell with a maximum of 11 colocalizations observed in one cell. The telomere associations declined in late S phase, to 11% of cells (336 cells from 3 experiments) and one to two per cell. hTR was also still found in or near Cajal bodies in most of these cells. hTR was not found at telomeres in G2 phase (our unpublished data). These results indicate that human telomerase RNA moves to telomeres during mid-S phase.

hTERT Also Associates with Telomeres and Nucleoli during S Phase

Telomerase requires both hTR and hTERT for function. Previous studies found that ectopically expressed GFP-hTERT fusion protein shows striking S-phase-specific intranuclear rearrangements (14, 15), but it was not found to localize to telomeres. Here, we have investigated the localization of endogenous hTERT protein.

We performed immunofluorescence using the 2C4 mAb against hTERT (20) and observed a punctate nuclear staining pattern in HeLa cells. The staining pattern described previously for this antibody in HeLa cells was more generally nucleoplasmic (20), so we examined the specificity of the pattern observed in our experiments (Figure 2.3A). We found that the more restricted staining pattern that we obtained was specific to hTERT. We knocked down hTERT expression in HeLa cells by RNA interference (20) and observed a marked decrease in the fluorescence signal intensity (including complete elimination of signal in some cells), indicating that the signal in the intranuclear foci corresponded to hTERT (Figure 2.3A). As an additional test, we

compared staining in IMR90 primary lung fibroblasts to IMR90 cells stably expressing hTERT from an exogenous construct (19). We observed some staining in the primary fibroblasts (Figure 2.3A), consistent with previous observations that normal cells express a low level of hTERT protein (20). The number and intensity of the foci was significantly greater in the cells expressing exogenous hTERT. Similar results were seen when hTERT was stably expressed in BJ cells (our unpublished data). Together, these results indicate that the 2C4 staining observed under these conditions is specific to hTERT and that hTERT is found in nucleoplasmic foci.

In synchronized cells, we found that hTERT (like hTR) localizes to nucleoli during the early stages of S phase (Figure 2.3B). Diffuse nucleolar localization of hTERT is detectable in 23% of cells at G1/S (429 cells analyzed from 2 separate experiments) and intensifies as cells enter early S, when the localization is apparent in 35% of cells (282 cells from 2 experiments) (Figure 2.3B). Like hTR, hTERT was observed in a subset of nucleoli (typically 1) within a cell. Nucleolar association of hTERT declined as cells progressed through S phase (9% of 236 cells in late S phase in 2 experiments).

Later in S phase, additional hTERT foci occurred, which corresponded to a subset of telomeres (Figure 2.3C). Colocalization of hTERT and telomeres (assessed by TRF1 [or TRF2; our unpublished data] antibody staining) occurred in a few (<5%) cells at G1/S (391 cells from 2 experiments). Localization of hTERT to telomeres peaked in mid-S phase when 24% of cells (463 cells from 2 experiments) were found to contain one to five colocalizations (Figure 2.3C).

Unfortunately, to date we have been unsuccessful in simultaneously analyzing hTERT and hTR (because of incompatibility of the IF and FISH protocols).

Independently, hTR and hTERT display remarkably similar temporal patterns of localization to telomeres and nucleoli during S phase (Figure 2.4). The results suggest an S-phase-specific mobilization of the components of the telomerase RNP to its functional destination, the telomere.

hTR and hTERT Are Found in Foci Adjacent to Cajal Bodies during S Phase

Our results indicate that hTR normally accumulates in Cajal bodies in HeLa cells and that hTERT does not. (Previously, we reported localization of YFP-hTERT to Cajal bodies in HeLa cells; [18]) However, in the current work, we did not detect significant accumulation of endogenous hTERT in Cajal bodies in either asynchronous or S-phase cells; hTERT colocalized with coilin in <5% of cells (Figure 2.4).) Although we did not observe endogenous hTERT in Cajal bodies, we did detect a specific accumulation of both hTERT and hTR in foci immediately adjacent to Cajal bodies during S phase. Figure 2.5 shows examples of the associations of hTR and hTERT with Cajal bodies observed in S-phase HeLa cells. The associations seem to represent one or two structures containing hTR or hTERT in direct contact with a Cajal body. In these cases, there is relatively little hTR (or hTERT) found in the core of the Cajal bodies. (The pattern does not represent simple pixel shifting relative to coilin data because the offsets occurred along multiple vectors within a single field and did not affect colocalizations in adjacent cells.) In some cases, particularly for hTR, it seems that the foci may be present at poles of the Cajal body (rather than in distinct adjacent structures) (e.g., Figure 2.5, row 2). In other cases, it is clear that the hTR or hTERT foci are distinct from the Cajal body (e.g., Figure 2.5, row 4). The timing and frequency with which hTR or hTERT are observed closely associated with Cajal bodies are similar to

telomere associations (Figure 2.4). At mid-S phase, localization to foci associated with Cajal bodies was found in 31% cells for hTR (698 cells analyzed from 5 separate experiments) and in 38% cells for hTERT (280 cells from 2 experiments). At least in the case of hTERT, there is also significant association in early S phase, suggesting that the peak of localization to Cajal body-associated foci occurs just before mid-S phase (Figure 2.4). It is not currently known whether the hTR or hTERT foci correspond with other, previously described Cajal body-associated nuclear bodies (26-30).

Discussion

Telomere synthesis is restricted to S phase in human cells. In this study, we show that the two key components of telomerase, telomerase RNA and the catalytic protein subunit hTERT, are targeted to telomeres specifically in S phase. Our study marks the first time human telomerase has been visualized at telomeres and suggests that the restriction of telomere elongation to S phase of the cell cycle is achieved by subnuclear trafficking of the telomerase RNP. Furthermore, our results indicate that multiple nuclear structures play roles in the regulated transport and biogenesis of telomerase.

Telomere Synthesis

Our finding that the recruitment of telomerase to telomeres is restricted to S phase (peaking in mid-S; Figures 2.2B, 2.3C, and 2.4) correlates well with the known timing of telomere elongation in human cells (12, 13). Strikingly, our data suggest that telomerase accumulates at only a subset of telomeres in a given cell (and in only a fraction of cells in a population) at any given time (Figures 2.2B, 2.3C, and 2.4). The

lack of detection of telomerase at some telomeres could certainly reflect further limitations of the experimental approach. However, this finding is also consistent with the idea that telomerase may not act on every telomere during every cell cycle, as has been demonstrated in yeast where only a small fraction of telomeres (~7%) are extended within a given cycle (31). Studies in both yeast and mammalian cells indicate that telomerase preferentially elongates the shortest telomeres in a population (31, 32). Alternatively, all telomeres may be extended during each cell cycle, but not simultaneously, such that telomerase is active at only a subset of telomeres at any given time point. It is known that chromosomes replicate at different rates during S phase (33, 34), and thus the timing of telomere synthesis could vary, for example, with the timing of replication-induced changes in chromatin structure and telomere accessibility at individual chromosomes.

Telomerase Trafficking Pathway

By following the subcellular localization of endogenous hTR and hTERT molecules throughout the cell cycle, we have obtained important insight into the pathway that telomerase may follow on its way to its site of action, the telomere (Figure 2.6). However, it is important to note that although the successful detection of hTR and hTERT at telomeres suggests excellent sensitivity, the results of our experiments do not preclude the presence of lower concentrations of hTR or hTERT in cellular compartments other than those identified, including the nucleoplasm. In addition, as discussed above, the various localization patterns described here are not observed in every cell in a synchronized population at a given time point, which may reflect technical

limitations or real differences in the timing or extent of telomere synthesis within and among individual cells.

Before S phase, during G1 (and also following S phase, in G2), hTR and hTERT are observed in separate intranuclear structures (Figure 2.6A). Telomerase RNA is present in Cajal bodies (Figure 2.1), consistent with previous findings (16, 18). In contrast, hTERT accumulates in distinct nucleoplasmic foci, which may represent previously unrecognized nuclear bodies or identified structures not previously known to contain hTERT (Figure 2.3). These findings suggest that the two key subunits of telomerase may be sequestered away from one another throughout most of the cell cycle.

Specifically during S phase, hTR and hTERT exhibit a dynamic redistribution and become targeted to common intranuclear sites. In early S phase, both hTR and hTERT can be found associated with nucleoli, although apparently not within a shared compartment (Figure 2.6B). hTR is present in Cajal bodies that seem to reside around the periphery of the nucleolus (Figures 2.1 and 2.2), whereas hTERT seems to be distributed throughout the interior of the nucleolus (Figure 2.3). Movement of Cajal bodies to and from nucleoli has been documented previously (25) and may account for the appearance of hTR at nucleoli in S phase. Intriguingly, we often find hTR or hTERT at a single nucleolus within a cell. Although it is well known that the nucleolus supports a number of functions beyond its conventional role in ribosome biogenesis (35-37), our results suggest a previously undescribed division of labor among nucleoli.

Beginning in early S phase and peaking at mid-S (Figure 2.6C), a novel pattern of hTR and hTERT localization emerges in foci that seem to be physically associated

with Cajal bodies (Figure 2.5). The hTERT found in Cajal body-associated foci may originate in nucleoli or come directly from the nucleoplasmic hTERT foci. The hTR foci may arise by segregation of hTR to one pole of a Cajal body, as is suggested by some of our data (e.g., Figure 2.5, row 2). Although we cannot demonstrate colocalization of hTR and hTERT in the Cajal body-associated foci (or at telomeres) for technical reasons, it seems possible (based on the similarity of the spatial and temporal patterns, and frequency of occurrence) that both components of telomerase are found together here (Figure 2.6C). Our analysis of hTERT suggests that localization to the Cajal body-associated foci precedes localization to telomeres, which peaks more distinctly in mid-S phase (Figure 2.4, see occurrence of Cajal body association at early S). We envision that Cajal bodies with a compartmentalized cargo of hTR and hTERT deliver telomerase to individual telomeres throughout the cell (Figure 2.6C).

In support of a role for Cajal bodies in the delivery of telomerase to telomeres, we have observed occasional colocalization of the Cajal body-associated hTR and hTERT foci with telomeres (our unpublished data), but the very low frequency of these associations (observed in ~2-3% of S-phase cells) suggests that the interactions with telomeres would be either transient or not preserved under our experimental conditions. In addition, live cell imaging has revealed that Cajal bodies undergo dramatic movements within the nucleus, including journeys across the diameter of the nucleus, fusion with other Cajal bodies, fragmentation into smaller bodies, and transient associations with nucleoli and specific chromosomal loci (23-25, 38). (In contrast, the majority of telomeres seem to be anchored to the nuclear matrix with limited capacity for migration; [39, 40]) Finally, an intriguingly similar cell cycle-regulated delivery of

transcription and processing factors to histone gene loci also seems to involve Cajal bodies and closely associated foci. Emerging evidence suggests that HiNF-P, a histone gene transcription factor, and p220/NPAT, an associated protein, colocalize at or near Cajal bodies in S phase, which localize to histone gene loci resulting in activation of histone gene transcription (27, 30, 41-43). Similarly, the RNA processing factors CstF and CPSF seem to move out of Cajal bodies into adjacent structures (termed cleavage bodies) that colocalize with histone gene loci in S phase (28).

Although neither the detailed localization of endogenous hTR and hTERT through the cell cycle nor the visualization of hTR and hTERT at telomeres has been described previously, some aspects of the pathway described in this work are supported by previous reports. The association of hTR with Cajal bodies in human cells throughout most of the cell cycle is consistent with the previous observations of our laboratory and others (16, 18). However, Jady et al. reported an increase in the brightness of hTR FISH signal in Cajal bodies (relative to nucleoplasm) in cells in S phase (16), and it is not immediately clear how this observation relates to the pathway defined here. Consistent with our findings, a small percentage of cellular hTR has been detected in biochemical fractions containing nucleoli (44), and hTR localizes to nucleoli (as well as Cajal bodies) when injected into Xenopus oocytes (45). In addition, ectopically expressed GFP-hTERT fusion proteins have been found to localize to nucleoli, and Yang et al. reported increased nucleolar association during S phase (14, 15). Our results place the previous observations firmly in the context of a cell cycle-regulated pathway.

Regulation of Telomerase Trafficking

Our results indicate that the transport of telomerase to telomeres is exquisitely regulated in the context of the cell cycle. On the basis of these findings, it will now be interesting to determine how movement of the RNA and protein subunits is linked to the cell cycle. One logical possibility is that some of the same kinases and phosphatases that drive other S-phase events (e.g., cdk2/cyclin A and cdc25) also modify telomerase subunits (and telomere binding proteins) to regulate molecular interactions and thereby influence telomerase trafficking (and telomere accessibility). In hTR, RNA elements termed the CAB box and H/ACA motif have been found to be important for localization of the RNA to Cajal bodies and nucleoli, respectively (16, 45), and the domains of hTERT that mediate nucleolar localization have been defined (15, 46). One would predict that these domains and proteins that interact with these domains could be modified to effectively regulate telomerase trafficking.

Telomerase Biogenesis

It is clear from our results that the trafficking of hTR and hTERT is regulated by the cell cycle. Interestingly, our findings suggest the possibility that the assembly of the telomerase enzyme may also be regulated to restrict telomere synthesis to S phase (i.e., the essential subunits may be compartmentalized away from each other as well as from their substrate). The detectable pools of hTR and hTERT are not found in common structures outside of S phase (Figures 2.1 and 2.3), suggesting that human telomerase is assembled specifically during S phase and disassembled (or destroyed in the case of hTERT; [20, 47]) after each cell cycle, perhaps during M phase when the telomerase

subunits do not seem to be associated with structures (Figure 2.1; our unpublished data).

If the biogenesis of telomerase is regulated by the cell cycle, our results suggest two likely sites for the assembly of the enzyme during S phase. hTR and hTERT are both found in foci associated with Cajal bodies and at telomeres in mid-S phase. The Cajal body-associated foci hold the potential for interaction with the SMN complex, a known RNP assembly factor that resides in Cajal bodies (48, 49). SMN is known to interact with telomerase and specifically to interact with GAR1 (an hTR-associated protein) and hTERT, suggesting that SMN may function in the biogenesis of telomerase (50, 51).

Our data are also consistent with the possibility that telomerase may assemble at the telomere. There is solid evidence that yeast telomerase is assembled at telomeres. In this case, the core components of telomerase, TLC1 (telomerase RNA) and Est2p (TERT) are constitutively present at telomeres, and assembly of active telomerase is regulated by an S-phase-specific recruitment of an essential telomerase subunit, Est1, to telomeres (52).

However, although telomerase activity is restricted to S phase in intact cells (12, 13), catalytically active telomerase enzyme (assessed by TRAP assay) can be extracted from both human and yeast cells at any stage of the cell cycle ([53] but see [54, 55]). It is not clear whether this extracted telomerase activity reflects enzyme present in cells or assembled from individual components after cell lysis and extract preparation (as has been demonstrated to occur in the case of another RNA-protein

complex; [56]). The potential regulation of telomerase biogenesis by the cell cycle will require further investigation.

Coupling telomerase trafficking to the cell cycle may have evolved in eukaryotes as an efficient mechanism to restrict the activity of telomerase to the period when chromosomes are replicated and to limit potentially deleterious activity of telomerase at nontelomeric sites (i.e., chromosome healing at double-stranded breaks) during the remainder of the cell cycle (57). Future research in the field will further delineate the intranuclear trafficking patterns of telomerase, define the molecular mechanisms of telomerase biogenesis and telomere recruitment, and determine how these processes are regulated by the cell cycle.

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Figure 2.1. Localization of human telomerase RNA in HeLa cells at various stages of the cell cycle. hTR (red, detected by FISH) and coilin (green, Cajal body marker protein detected by IF) were analyzed by fluorescence microscopy in cells 0 (G1/S), 4 (mid-S), 8 (G2), 10 (M), 12 (early G1), and 15 (mid-G1) hours after release from double thymidine block. Differential interference contrast (DIC) panels show differential interference light microscopy data. DAPI panels shows DNA staining. Merge panels show superimposition of hTR and coilin fluorescence data (yellow indicates overlap of signal). Arrows in mid-S merge panel indicate hTR foci that do not colocalize with coilin.

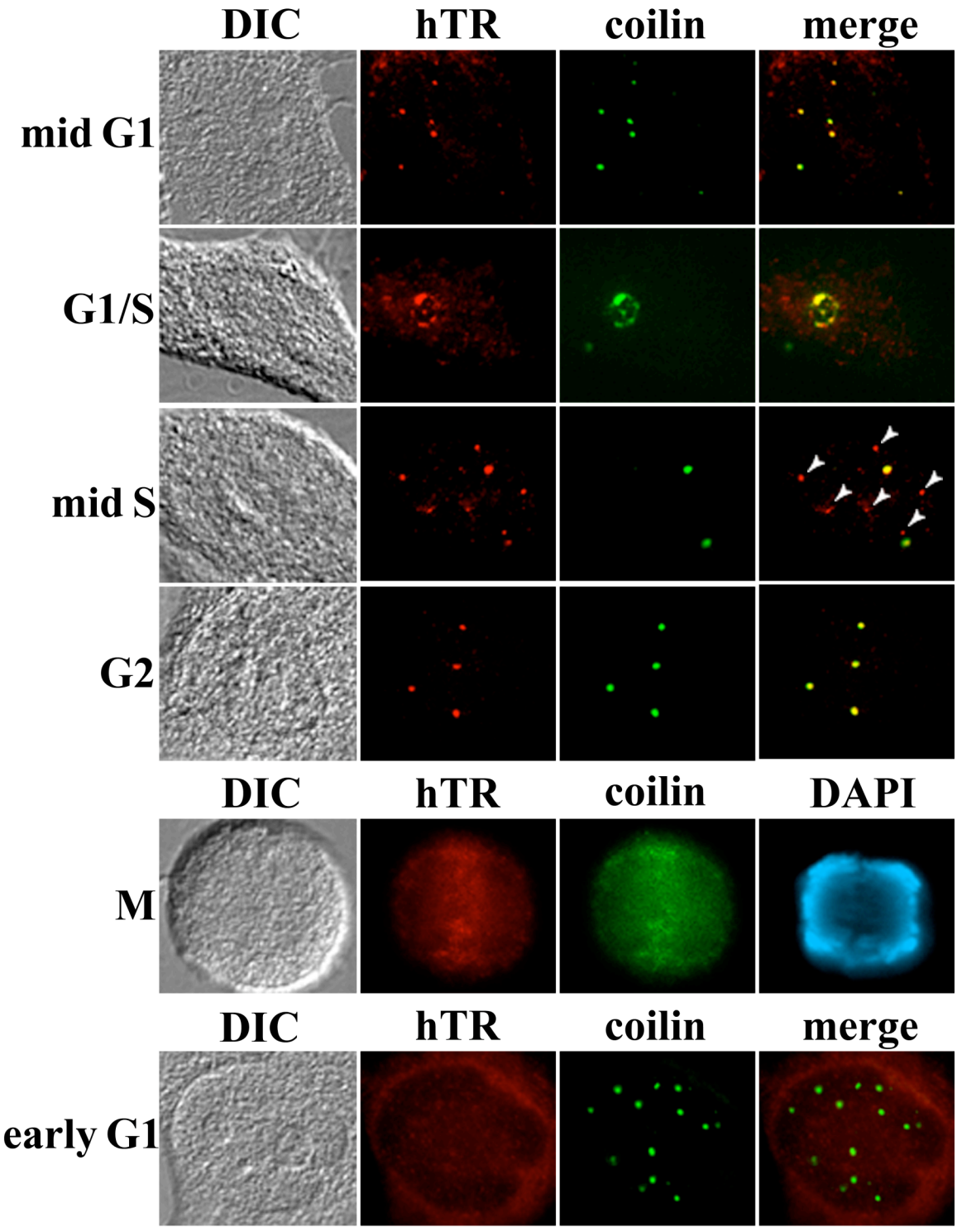

DIC
hTR
coilin
merge
mid G1
G1/S
mid S
G2
DIC
hTR
coilin
DAPI
M
DIC
hTR
coilin
merge
early G1

Figure 2.2. Human telomerase RNA is found at the periphery of nucleoli and at telomeres during S phase. *(A) hTR and coilin seem to be associated with the surface of nucleoli during early S phase.* The localization of hTR (red, detected by FISH) is superimposed on coilin (green, detected by IF, 1) or cellular architecture (visible by DIC light microscopy, 2 and 4) in indicated panels. The cells shown are in early S phase. Arrowheads denote nucleoli exhibiting apparent peripheral or surface hTR signals. *(B) hTR associates with telomeres during mid-S phase.* hTR (red, detected by FISH) and telomeres (green, detected by TRF1 IF or telomere FISH as indicated) were analyzed in mid-S-phase cells. DAPI panel shows DNA staining. BrdU panel shows a mid-S-phase pattern. Arrows in merge panels indicate foci where both hTR and telomeres are present.

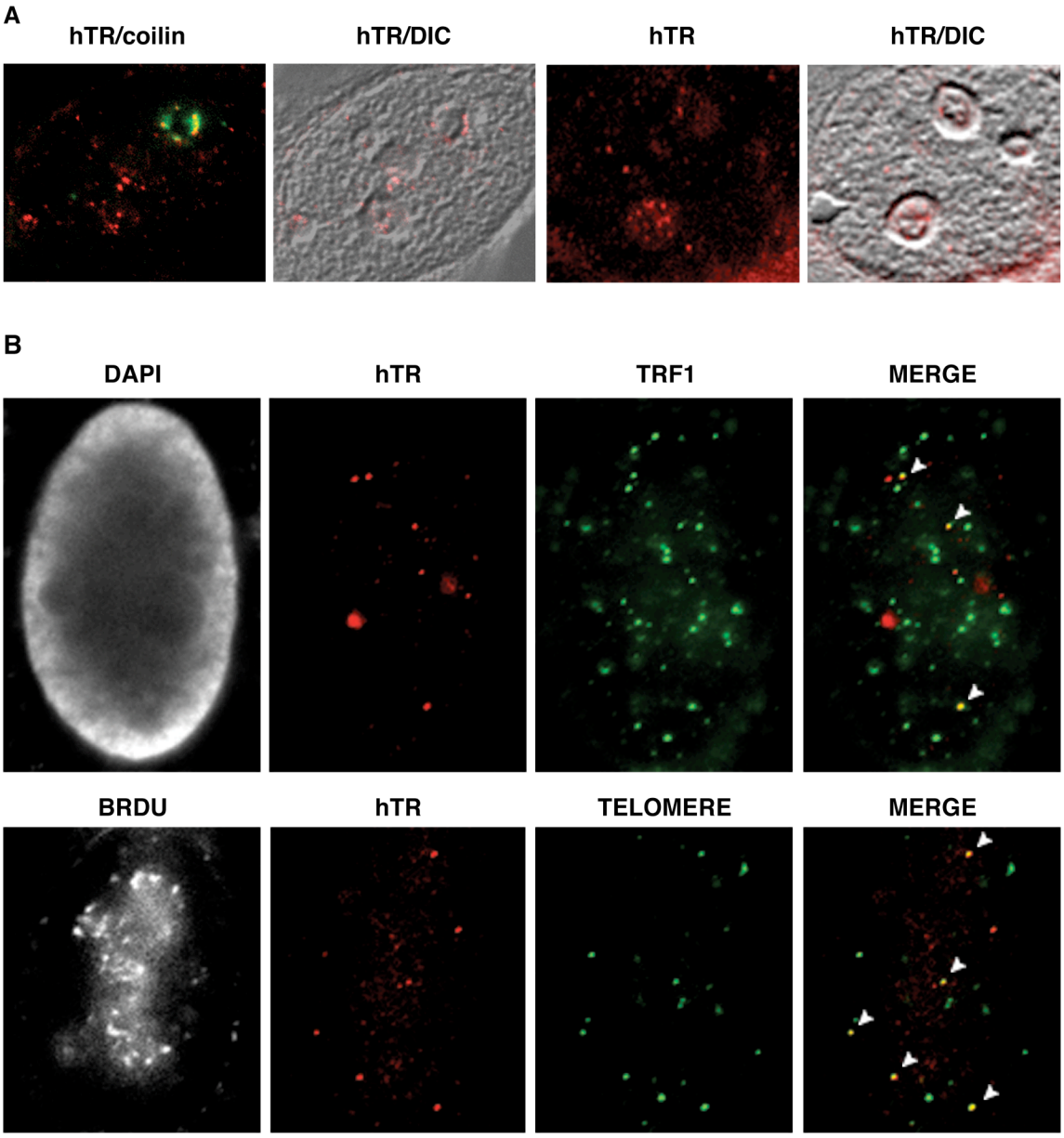

A
hTR/coilin
hTR/DIC
hTR
hTR/DIC
B
DAPI
hTR
TRF1
MERGE
BRDU
hTR
TELOMERE
MERGE

Figure 2.3. Cellular localization of human telomerase reverse transcriptase. *(A) Intranuclear foci recognized by hTERT antibody 2C4 are specific to hTERT.* The panels show 2C4 immunostaining of HeLa cells and HeLa cells within which hTERT was knocked down by RNA interference (-hTERT) and of IMR90 primary fibroblasts and an IMR90 strain that overexpresses hTERT (+hTERT). Data in each pair of panels were normalized to allow visual comparison. *(B) hTERT is found in nucleoli in early S-phase cells.* The localization of hTERT (red, detected by IF) is superimposed on cellular architecture visible by DIC light microscopy in merge panels. *(C) hTERT is found at telomeres in mid-S-phase cells.* hTERT (red) and TRF1 (green) localization is shown. Arrowheads indicate representative foci where both hTERT and TRF1 are present.

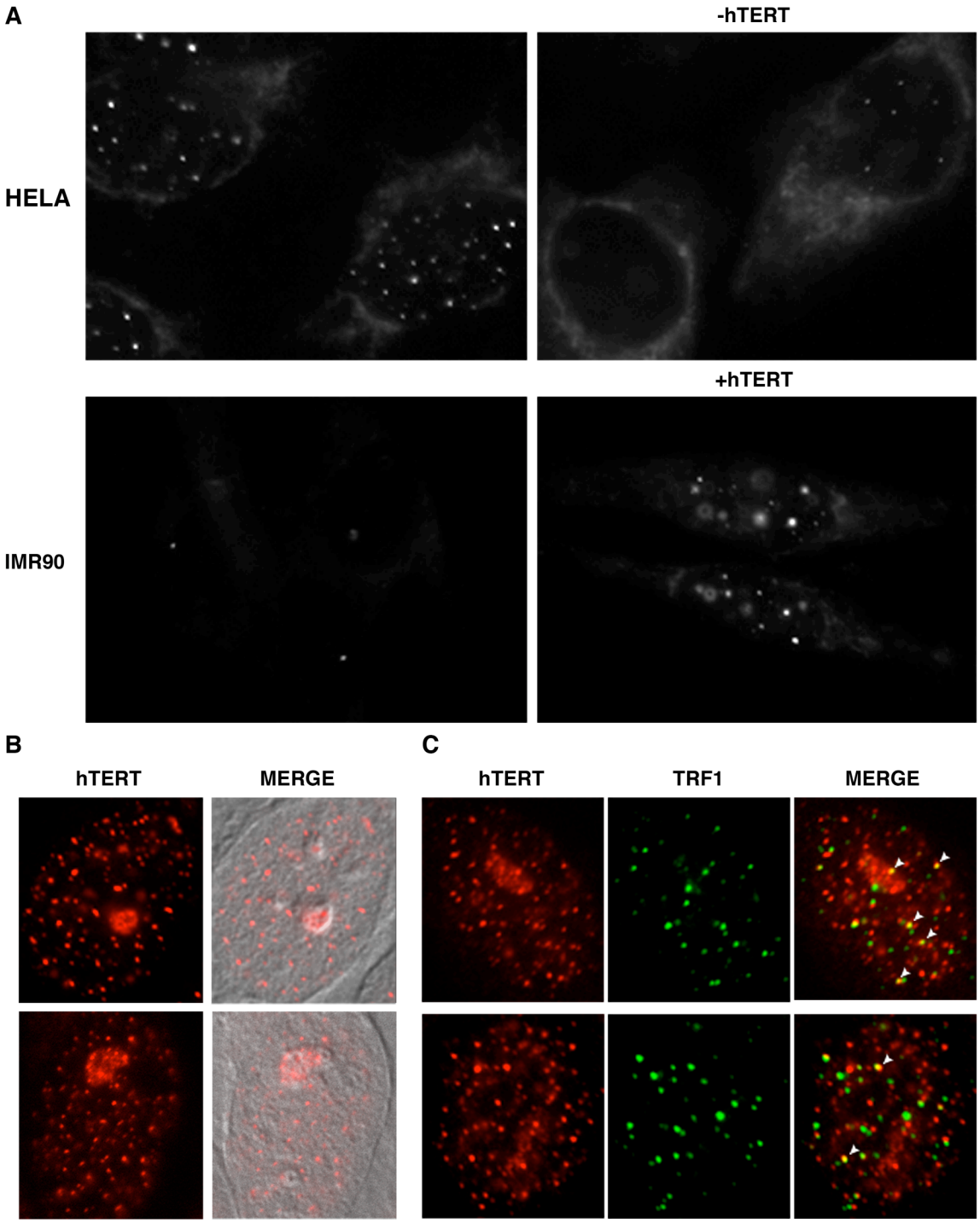

A
-hTERT
HELA
+hTERT
IMR90
B
hTERT
MERGE
C
hTERT
TRF1
MERGE

Figure 2.4. Temporal patterns of association of human telomerase RNA and telomerase reverse transcriptase with nuclear structures during S phase in HeLa cells. The percentage of cells in which hTR (A) and hTERT (B) was found associated with each structure in G1/S, early S, mid-S, late S, and S/G2 phase cells is indicated as follows: telomeres (●) and nucleoli (hTR peripheral and hTERT internal) (▲) on left axis; colocalization with Cajal bodies (◆) and Cajal body-associated foci (■) on right axis (note difference in scale of axes). Each point plotted represents an average of at least 158 (and as many as 698) total cells counted from at least two (and as many as 6) experiments, with the exception of the S/G2 time point for hTERT, which represents data from a single experiment.

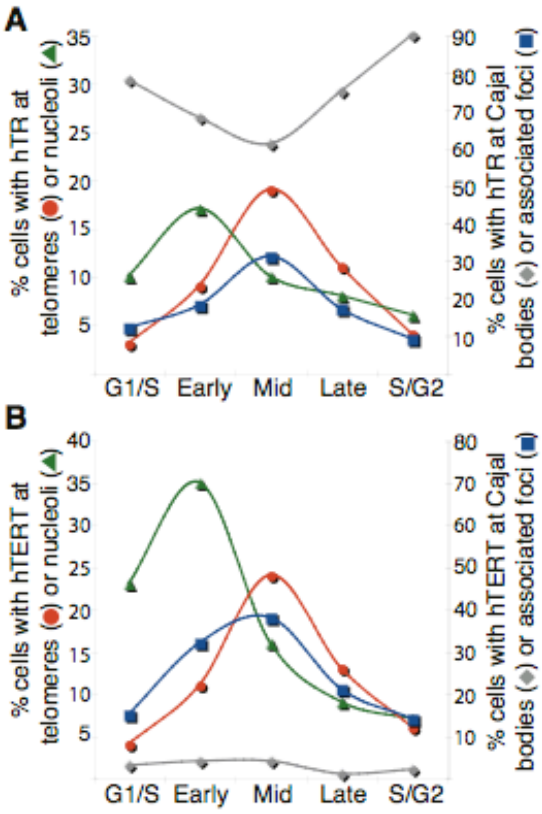

A
% cells with hTR at telomeres (●) or nucleoli (▲)
% cells with hTR at Cajal bodies (◆) or associated foci (■)
35
30
25
20
15
10
5
90
80
70
60
50
40
30
20
10
G1/S
Early
Mid
Late
S/G2
B
% cells with hTERT at telomeres (●) or nucleoli (▲)
% cells with hTERT at Cajal bodies (◆) or associated foci (■)
40
35
30
25
20
15
10
5
80
70
60
50
40
30
20
10
G1/S
Early
Mid
Late
S/G2

Figure 2.5. Human telomerase RNA and telomerase reverse transcriptase are found in foci adjacent to Cajal bodies during S phase. Coanalysis of coilin (blue) and hTR (red) or hTERT (red) in early or mid-S-phase HeLa cells is shown. Merge panels show superimposition of coilin and hTR or hTERT signals. Insets show enlargements of close associations of distinct coilin and hTR or hTERT foci (indicated by arrowheads in merge panel).

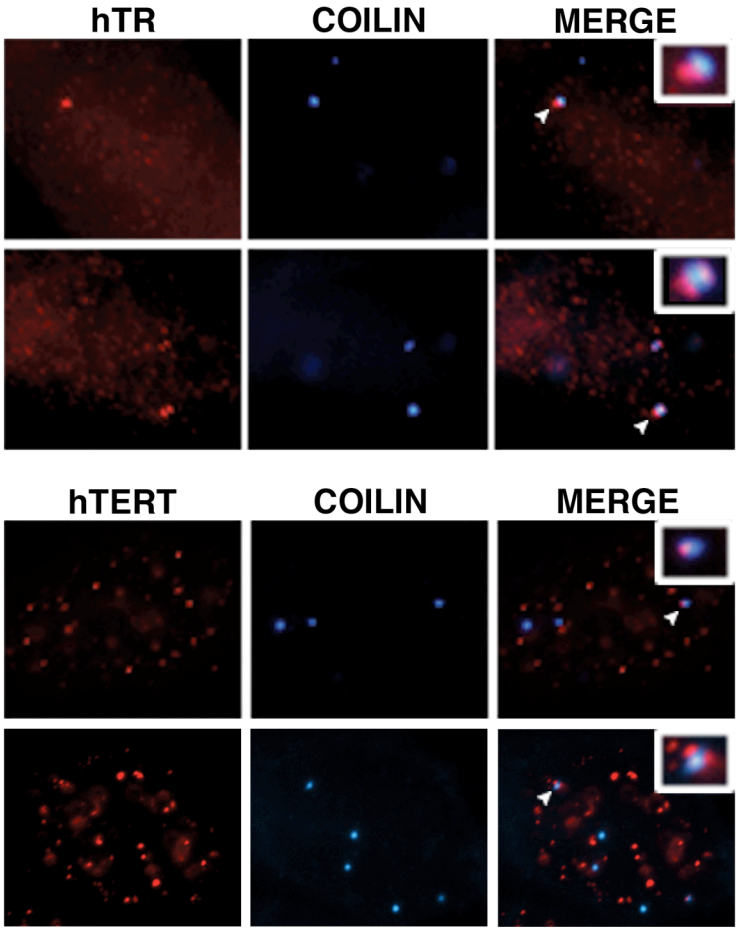
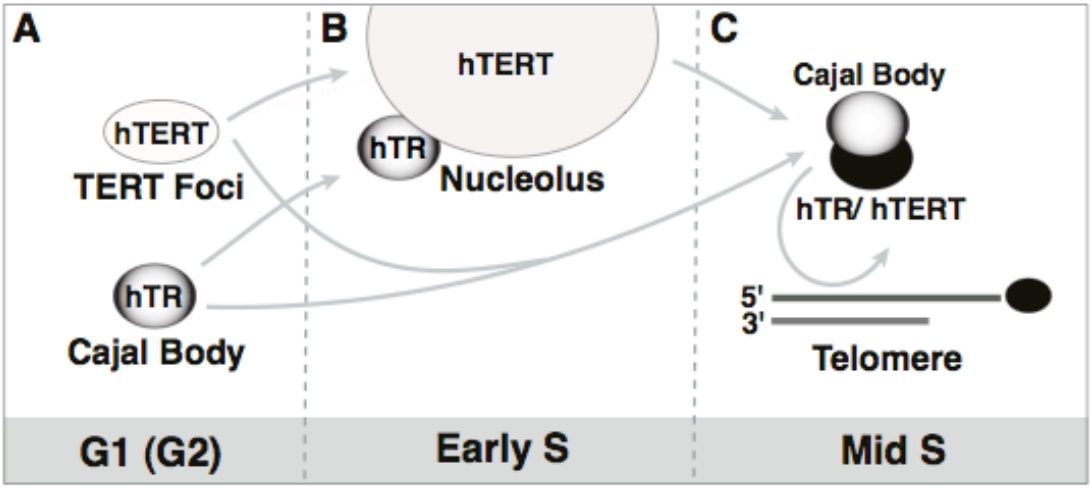

hTR
COILIN
MERGE
hTERT
COILIN
MERGE

Figure 2.6. Model for cell cycle-regulated trafficking of telomerase subunits to telomeres during S phase. The predominant phase-specific localization of hTR and hTERT is shown for G1 (A), early S (B), and mid-S (C). The arrows indicate possible trafficking pathways accounting for the observed localizations. See text for details.


A
B
C
hTERT
hTERT
TERT Foci
hTR
Nucleolus
Cajal Body
hTR/ hTERT
hTR
Cajal Body
5'
3'
Telomere
G1 (G2)
Early S
Mid S

Chapter 3

hTERT Expression Drives Telomerase RNA Localization to Cajal Bodies and Telomeres[1]

[1]Rebecca L. Tomlinson, Eladio B. Abreu, Claus Azzalin, Gael Cristofari, Christopher M. Counter, Hinh Ly, Joachim Lingner, Rebecca M. Terns and Michael P. Terns, to be submitted to the *Journal of Cell Biology*.

Abstract

Human telomerase RNA (hTR) is housed within intranuclear structures termed Cajal bodies throughout most of the cell cycle in telomerase-positive cancer cells. During S phase, as telomeres are being synthesized, hTR is recruited to subsets of these telomeres. Here we present data that the trafficking of hTR to these two nuclear locales is dependent on expression of the telomerase reverse transcriptase (hTERT) protein. Knockdown of hTERT in cancer cells leads to a loss of hTR from both Cajal bodies and telomeres. Furthermore, expression of hTERT in telomerase-negative cells (including primary cell lines and ALT cancer lines) induces hTR to localize to Cajal bodies and telomeres. The reciprocal relationship holds true as hTR expression promotes the association of hTERT with both Cajal bodies and telomeres. Our results show that hTR and hTERT influence the trafficking of one another and suggest that interaction of these two key telomerase components is required for their recruitment to the telomere.

Introduction

Telomerase is the ribonucleoprotein (RNP) enzyme that utilizes a short motif within its integral RNA subunit (telomerase RNA, or hTR) to synthesize the DNA repeats that make up the telomere (1). In humans, the enzyme's activity is restricted to early prenatal development, synthesizing enough telomeric repeats to last throughout the life of the organism (2, 3). Most adult somatic tissues contain low or undetectable levels of telomerase activity, leading to shortening of the telomeres and a limited replicative capacity. Although relatively inactive in the majority of adult somatic tissues, telomerase is reactivated in over 90% of human cancers, and telomere maintenance by

telomerase is critical for the continued proliferation of these cells (4). The presence of telomerase activity correlates with expression of the catalytic telomerase reverse transcriptase (hTERT) subunit, indicating hTERT is the limiting component for enzyme assembly and function (5).

Previously, our lab and others reported the development of a fluorescence *in situ* hybridization (FISH) procedure specific for the detection of hTR in human cell lines (6-9). We determined that hTR localizes to intranuclear structures called Cajal bodies in telomerase-positive cancer cells (6, 8). A dynamic redistribution of hTR from the Cajal bodies to the telomeres occurs during S phase of the cell cycle in these cancer cells (7, 9), corresponding with the timing of telomere synthesis in these cells (10, 11). Our data together with the results from the Kiss lab suggest that Cajal bodies serve as sites of telomerase maturation and assembly, and act to deliver active telomerase to the telomere. Though hTR is expressed in normal cell lines, it does not localize to Cajal bodies (or any other intranuclear structure), and is instead found diffusely distributed throughout the nucleoplasm (6).

Here, we utilize the localization difference between normal and cancer cells in an effort to better understand the trafficking of hTR. We systematically examined a variety of factors that differ between normal and cancer cells to identify determinants of hTR localization. Our results indicate that hTERT expression is essential for localizing hTR to both Cajal bodies and telomeres. Knockdown of hTERT expression (by RNAi) in HeLa cancer cells leads to a reduction or elimination of hTR within Cajal bodies and at telomeres; instead hTR becomes diffusely localized throughout the nucleus. Stable expression of hTERT in ALT (alternative lengthening of telomeres) cells (which are

normally devoid of the protein) leads to hTR localization within Cajal bodies and to telomeres. Ectopic expression of hTERT in normal cells leads to an S phase-specific recruitment of hTR to the telomere (the site of telomerase function). Taken together, our results suggest that the intranuclear trafficking of telomerase RNA depends on expression of hTERT. Furthermore, we find that localization of hTERT to both telomeres and Cajal bodies is triggered by expression of hTR, indicating both components of the telomerase RNP influence the trafficking of each other.

Results

hTERT expression leads to the formation of hTR foci in normal cells

Recently we and others reported the S phase-specific trafficking of telomerase components (7, 9). Our data suggest that hTR and hTERT are transported from separate sites (where they reside throughout most of the cell cycle; hTR within Cajal bodies and hTERT in as yet unidentified nuclear foci), transit through nucleoli and Cajal body-associated foci before ultimately reaching their functional destination, the telomere (7). In order to better understand the determinants which influence this trafficking pathway, we have taken advantage of the fact that normal cells do not exhibit hTR foci, but still express the RNA. Here, we have examined factors that differ between normal and cancer cells as a means of identifying specific determinants that effect the localization of hTR.

One key difference between normal and cancer cells is the degree to which they express hTR; on average cancer cells express two-fold higher levels of hTR than normal cells do (12, 13). To see whether expression of higher levels of hTR in normal

cells could lead to induction of hTR foci, we transiently expressed a construct encoding hTR (14) in IMR90 primary lung fibroblasts. This plasmid also encodes a GFP (green fluorescent protein) reporter gene, allowing us to determine which cells in a population were overexpressing hTR (i.e. those that have been transfected). Following transfection, no hTR foci were observed in the GFP-positive cells; in fact, hTR exhibited the same diffuse localization pattern in transfected and untransfected cells (Figure 3.1A; transfected cell displays GFP signal). Stable expression of this same hTR construct in VA13 cells, a cell line normally devoid of hTR transcripts (15), led to higher levels of hTR expression than in HeLa cells (Figure 3.1B). In these stable lines, no hTR foci were observed despite the high levels of hTR expressed (Figure 3.1A), further confirming that high hTR levels do not trigger the localization of hTR into subnuclear structures.

In addition to the low hTR levels, primary cell lines lack well-formed Cajal bodies. In a given population of primary cells, less than 10% of cells will organize their coilin into Cajal bodies (16). In contrast, over 90% of cancer cell lines will have at least one Cajal body per nucleus (16). To test whether the presence of Cajal bodies influenced the localization of hTR, we induced Cajal body formation in human mammary epithelial (HME) cells (Figure 3.1C) and IMR90 cells (data not shown) by culturing the cells at 32°C instead of at 37°C for 24 hours (17). MCF7 breast cancer cells were analyzed at both temperatures to ensure the decreased temperature did not negatively effect hTR localization (Figure 3.1C). Growing cells at 32°C instead of 37°C led to induction of Cajal body formation (monitored by coilin IF) in over 40% of normal cells examined. Despite this increase in the number of cells with Cajal bodies, no change was seen in

hTR localization in the HME and IMR90 cells grown at 32°C (Figure 3.1C and data not shown), indicating that the presence of a well-formed Cajal body was not sufficient to bring about hTR localization to that Cajal body (or to any subnuclear structure).

In order to further test whether high hTR levels and Cajal bodies could affect hTR localization, we examined hTR localization in the ALT (alternative lengthening of telomeres) cell lines IIICF-T/C3 and GM847. ALT cells are immortalized cell lines that do not have active telomerase and instead maintain their telomeres by a telomerase-independent mechanism (18). We found that these cells, unlike normal cells, contained well-formed Cajal bodies in roughly 1/3 of the total cells in a population. As can be seen in Figure 3.1D, hTR does not accumulate within these Cajal bodies in either IIICF-T/C3 or GM847 cells. Unlike normal cell lines, these two cell lines expressed levels of hTR comparable to the commonly used cancer line, HeLa (Figure 3.1B and see [15]), which further confirmed high levels of hTR expression and the presence of well-formed Cajal bodies are not enough to influence the localization of hTR.

Given that low hTR levels and the absence of Cajal bodies were not contributing to the lack of hTR foci in normal cells, we wanted to examine other factors that may influence the localization pattern of hTR. Three gene products are known to transform primary cells into an oncogenic state: the large T antigen of SV40 (transforms the cells; inactivates p53 and Rb pathways), hTERT (for telomere maintenance and cellular immortality) and oncogenic Ras (required for oncogenic transformation) (19, 20). We examined (by hTR FISH) whether expression of any of these genes could induce localization of hTR into subnuclear foci alongside of control MCF7 breast cancer (Figure 3.2, row 1) and human mammary epithelial (HME) primary (Figure 3.2, row 2) cell lines.

In HME cells that express all three genes (HME-hTERT/large T/onco. Ras), hTR was found in nucleoplasmic foci in approximately one third of cells examined (Figure 3.2, row 3). To test which of these factors was responsible for localization of hTR to foci, we performed hTR FISH on a series of HME cell lines, each line expressing one or more of the three defined genetic elements (20). We found that co-expression of large T and oncogenic Ras (or large T alone) had no effect on hTR localization, as no hTR foci were exhibited in cells expressing just these two transgenes (Figure 3.2, row 4 and data not shown). Expression of hTERT alone was responsible for the emergence of hTR foci (Figure 3.2, row 5), contributing to the presence of hTR foci in one third of cells examined (a similar percentage to the HME cells expressing all 3 transgenes). Similarly, in cells expressing both large T and hTERT, approximately one third of cells display hTR foci (data not shown).

hTERT expression is essential for localization of hTR to Cajal bodies

Since hTERT was sufficient to induce hTR foci in normal cells, we set about to determine if any of these foci were Cajal bodies. Cajal body induction (from culturing at 32°C) in the HME-hTERT cell line resulted in approximately half of the hTR foci colocalizing with coilin (Figure 3.3), indicating that many of the foci are in fact Cajal bodies. Similar results were obtained upon Cajal body induction in IMR90-hTERT cells (data not shown). Additionally, we tested the effect of hTERT expression on hTR localization in the ALT line GM847. Upon stable expression of hTERT in the GM847 cells, hTR localized to Cajal bodies in approximately 30% of cells (Figure 3.3); every time a Cajal body was present, hTR was found localized within that Cajal body.

To further test the significance of hTERT expression on the localization of hTR, we knocked down expression of hTERT in HeLa and MCF7 cancer cells using RNA interference (RNAi). By expressing a short hairpin RNA (shRNA) against the hTERT message (21), we significantly lowered hTERT protein levels, thus mimicking the situation of a normal cell. To ensure efficient knockdown of hTERT, we performed immunofluorescence (IF) with an antibody against hTERT (21) on cells transfected with the hTERT shRNA and compared the signals to untransfected cells and cells transfected with an empty vector. In cells transfected with the empty vector (and in untransfected cells), 5-20 small foci were observed in over 90% of nuclei (Figure 3.4A, top left set of panels and data not shown). When the hTERT shRNA was expressed, the majority of cells (70-84%) displayed no hTERT foci (Figure 3.4A).

We subsequently performed hTR FISH and coilin IF on cells expressing the hTERT shRNA and found a similar trend; 72-84% of cells displayed no accumulation of hTR within Cajal bodies (or at any other nuclear site, including telomeres) (Figure 3.4A). The average hTR signal intensity in the cells with remaining hTR foci was substantially weaker than that of the untransfected and empty vector controls, where hTR is found in Cajal bodies in over 80% of cells (Figure 3.4A, compare top right row of panels to the bottom right row). The reduction in the number of cells with hTR foci (and the drop in hTR signal intensity of those cells that do have foci) was not due to a decline in hTR levels in the cells that express the hTERT shRNA (Figure 3.4B). Instead, the loss of hTR localization to Cajal bodies (and telomeres) seemed to be due to a drop in expression of hTERT, indicating that hTERT was both necessary and sufficient for the localization of hTR within Cajal bodies.

Ectopic expression of hTERT in normal cells leads to an S phase-specific recruitment of hTR to the telomere.

While hTERT expression was sufficient to induce hTR localization to nuclear foci in normal cells (Figure 3.2), only around one third of cells displayed these foci. This was in sharp contrast to cancer cells (such as MCF7, see Figure 3.2, row 1), in which greater than 80-90% of cells exhibited hTR foci. Our lab and others have recently shown that hTR exhibits a dynamic change in localization during S phase of the cell cycle (7, 9). To test if there was an S phase specificity to the observance of hTR foci in the hTERT-expressing normal cells, we performed hTR FISH in combination with BrdU (5-Bromodeoxyuridine) labeling on normal cells (IMR90 and HME) and normal cells that ectopically express hTERT (IMR90-hTERT and HME-hTERT) (Figure 3.5A and data not shown). BrdU is a thymidine analog that is incorporated into the DNA of cells undergoing DNA replication (i.e. cells in S phase of the cell cycle). A cell in S phase exhibits one of several BrdU staining patterns, depending on which stage of S phase (early, mid, or late) the cell is in (22). For cells that ectopically express hTERT, we discovered that the majority of the cells that contained hTR foci were in fact in S phase (Figure 3.5A, top 2 rows of panels). No hTR foci were found in IMR90 (or HME) cells in S phase (or any other stage) of the cell cycle (Figure 3.5A, bottom row and data not shown).

To further verify that accumulation of hTR in normal cells that ectopically express hTERT was related to cell cycle stage, we synchronized IMR90 and IMR90-hTERT cells at the G1/S transition using a double thymidine block. At various time points following release from that block, we analyzed the cells by hTR FISH and BrdU labeling (to

determine synchronization efficiency and identify the S sub phase as early, mid, or late). Enriching for cells in S phase of the cell cycle led to an increase in the number of IMR90-hTERT cells with hTR foci, with a peak occurring during mid-S phase of the cell cycle (4h post release from the block) (Figure 3.5B). During mid-S phase, 66% of cells contained hTR foci (129 cells out of 196 total, data compiled from 2 separate experiments), compared with only 34% of asynchronous cells (49 cells had hTR foci out of 144 total, data compiled from 2 separate experiments). Synchronization did not induce the formation of hTR foci in IMR90 cells (data not shown), further indicating that hTERT expression was key for formation of these foci.

To see whether hTR was found at the telomeres in these hTERT-expressing normal cells, we performed hTR FISH in combination with immunofluorescence using antibodies against the telomere binding proteins TRF1 or TRF2 on IMR90-TERT and HME-hTERT cells (Figure 3.6A). We found that hTR colocalized to a subset of telomeres in 65-70% of the cells that have hTR foci (equivalent to ~20% of the total population of cells). Localization of hTR to the telomere only occurred in BrdU-positive cells, indicating that it was an S phase-specific event. In the S phase synchronized IMR90-TERT cells, greater than 90% of the hTR foci were found at a telomere. In fact, the percentage of hTR-telomere associations at each time point after release from a double thymidine block closely matched the graph seen in Figure 3.5B. hTR was not found at the telomeres in normal (or ALT) cells (data not shown), indicating hTERT expression was required for the localization event.

To further assess the importance of hTERT expression in localizing hTR to the telomere, we examined hTR localization in VA13 ALT cells that stably expressed both

hTR and hTERT. As described above, VA13 cell lines are normally devoid of both hTR and hTERT (15) and expression of hTR alone in VA13 cells resulted in no hTR foci (see Figure 3.1A). In striking contrast, stable expression of hTERT in these cells resulted in numerous hTR foci, all of which coincided with a telomere (as marked by TRF2 staining) (Figure 3.6B). Additionally, we did not detect hTR at the telomere in any of the HeLa cells in which hTERT expression was knocked down (Figure 3.4A and data not shown), further highlighting the importance of hTERT expression for trafficking of hTR to the telomere. Taken together, our data suggest that normal and cancer cells use a similar pathway to regulate access of telomerase to the telomere and that hTERT expression is sufficient to localize hTR to its functional site.

hTR expression triggers localization of hTERT to Cajal bodies and telomeres

Since hTERT influences the localization of hTR to both telomeres and Cajal bodies, we asked if the reciprocal relationship existed, and tested whether hTR expression influenced the localization of hTERT. To see the effects of hTR expression on the association of hTERT with both telomeres and Cajal bodies, we transiently transfected HeLa cells with vectors encoding for hTERT and hTR or empty vector controls and examined the localization of hTERT with relation to TRF2 (marker for telomeres, Figure 3.7) and coilin (marker for Cajal bodies, Figure 3.8).

As can been seen in Figure 3.7A, few nuclear hTERT foci were seen in cells transfected with the empty vectors; and these foci did not significantly overlap with telomeres. Upon overexpression of hTERT only in the HeLa cells, we began to see a diffuse staining for the hTERT protein, with a few instances of overlap of hTERT and telomere signals in most cells (Figure 3.7A, middle set of panels). When both hTR and

hTERT were transiently overexpressed in the HeLa cells (Figure 3.7A, bottom set of panels), the colocalizations between hTERT and TRF2 were more evident and we saw a greater number of hTERT foci at telomeres in each cell, indicating that increasing hTR levels significantly enhanced the association of hTERT with telomeres.

This dependency of hTERT localization to telomeres on hTR expression was verified by chromatin immunoprecipitation (ChIP) analysis (Figure 3.7B). hTERT antibodies immunoprecipitated more telomeric DNA in cells transfected with both hTR and hTERT (Figure 3.7B, lane 4) than cells expressing only one telomerase component (and empty vector cells), indicating hTR expression is enhancing the association of hTERT with the telomeres. A slight increase (over the empty vector control) in the amount of hTERT bound to telomeric chromatin was seen in cells overexpressing hTERT only, indicating the endogenous hTR (which is normally in excess of hTERT in the cell [13]) is enough to increase this interaction between hTERT and the telomere. The large increase in hTERT at telomeres exhibited by the cells expressing both hTR and hTERT was due to the increase in hTR expression (and not to higher levels of hTERT protein in the doubly transfected cells), as similar amounts of hTERT protein are expressed in the two sets of cells (Figure 3.7C).

We also examined whether hTERT was found in Cajal bodies in any of the transfected cells (Figure 3.8). Previously we found no significant instances of endogenous hTERT colocalizing with coilin, the marker protein of Cajal bodies (7), but have seen some association of exogenous YFP-tagged hTERT with Cajal bodies (6). Here, we overexpressed untagged hTERT in the absence or presence of exogenous hTR and assessed the role of hTR in localizing hTERT to Cajal bodies. As expected,

no significant colocalization of hTERT with Cajal bodies was seen in cells transfected with empty vectors (Figure 3.8, upper panels). Likewise, transient expression of hTERT alone did not lead to significant colocalization between hTERT and coilin (Figure 3.8, middle panels). However, when both hTR and hTERT were expressed in the cells, we saw a dramatic increase in the number of cells with hTERT in Cajal bodies (Figure 3.8, lower panels); indicating hTR expression also enhanced the localization of hTERT to Cajal bodies. It is worth noting that the number of Cajal bodies present in cells overexpressing both hTR and hTERT was much higher than what was observed for the empty vector control cells and the hTERT only cells. Taken together, this data indicates that hTERT trafficking is influenced by the presence of hTR.

Discussion

Despite the importance of human telomerase in chromosome maintenance, cellular immortalization, and oncogenesis, little is known about the essential steps in the generation of the enzyme from individual RNA and protein components and how the enzyme is recruited to its site of action, the telomere. Formation of telomerase enzyme depends minimally upon the intracellular interaction of hTR and hTERT and these subunits are initially synthesized in separate cellular compartments (nucleus and cytoplasm, respectively). In this study, we obtained evidence that the core subunits of the human telomerase holoenzyme (i.e. hTR and hTERT) influence the intranuclear trafficking of each other to both Cajal bodies (proposed sites of telomerase biogenesis and telomere delivery) as well as telomeres (sites of telomerase function). Our results suggest that the intranuclear trafficking of the key components of telomerase are tightly

coupled to their potential for interaction/assembly with one another into a functional telomerase enzyme.

Trafficking to telomeres

Previously, we and others reported that both hTR and hTERT are specifically recruited to a subset of telomeres in cancer cells during S phase of the cell cycle (7, 9), peaking at the time when telomeres are replicated (and more than likely synthesized) (10, 11, 24). In this work, we extend this finding and show that hTERT expression is both sufficient to induce hTR localization to telomeres (Figure 3.6), and necessary for this localization to occur (Figure 3.4A and data not shown).

Interestingly, when we ectopically express hTERT in normal cells, hTR is found at a fraction of telomeres roughly in roughly 20% of the cells in a given asynchronous population, which is significantly higher than the percentages observed for cancer cells (in which ~7% of cells display hTR at telomeres [7]). This translates into an increased number of cells with hTR-telomere associations throughout all stages of S phase. The timing of this localization event peaks during mid-S phase (analogous to the timing observed in HeLa cancer cells [7]). Unlike the cancer cells, the peak is not as sharply defined, as more of the hTERT-expressing normal cells in early and late S phase display hTR foci at the telomere when compared to the HeLa cells (see Figure 3.5B for an approximation of the distribution in hTERT-expressing normal cells). The ectopic expression of hTERT in these normal cells leads to higher levels of hTERT when compared with the endogenous levels of hTERT found in a cancer cell line (such as HeLa). Perhaps these high levels of hTERT result in increased trafficking of hTR to telomeres. Similarly, increasing hTERT levels in HeLa cells leads to an increase in the

number of cells with hTR-telomere associations (data not shown). In a recent report, Cristofari and Lingner proposed a model whereby increased levels of telomerase led to increased telomere length in a mechanism dependent on telomerase concentration (25). Taken with our data, it suggests that increasing levels of telomerase leads to a higher rate of association with the telomeres, which, in turn, leads to higher rates of telomere synthesis.

The rise in hTR-telomere associations can manifest itself in two ways: the first (as described above) would be for more cells to display hTR at the telomere, the second would be for there to be a greater number of hTR-telomere colocalizations per cell. For the most part, when hTERT is ectopically expressed, we find that hTR is found at a small subset of telomeres in any given cell (Figure 3.6A), further supporting the notion that not every telomere is extended at a given time (7, 26). The telomeres that recruit telomerase are more than likely the shortest telomeres in the population, as telomerase has been shown to preferentially elongate short telomeres first (26-29). However, when we increase the levels of both hTR and hTERT (as in the VA13+hTR+hTERT cells), we see a dramatic increase in the number of hTR-telomere colocalizations per cell (as well as in the number of cells with such colocalizations) (Figure 3.6B). A similar effect is seen upon overexpression of both components in HeLa cells on the localization of both hTR and hTERT to telomeres (Figure 3.7 and data not shown). Our results show that the levels of the two main subunits of the telomerase RNP influence the number of telomeres that can (and do) recruit telomerase at any given time in the cell cycle. Lower levels of each component could render telomerase more sensitive to detection of

telomere length, ensuring that the shortest telomeres are preferentially elongated in a given round of telomere synthesis.

Trafficking to Cajal bodies

Cajal bodies are small, spherical subnuclear organelles that participate in various steps of RNP biogenesis (30, 31). Cajal bodies have been implicated in aspects of telomerase biogenesis as well. hTR resides within this nuclear structure throughout much of the cell cycle, and various steps in the posttranscriptional modification and processing of the RNA likely occur within Cajal bodies. For example, hTR carries a trimethyl guansoine (TMG) cap, and it is likely this modification is carried out in Cajal bodies by the methyltransferase Tgs1 (8, 32) . Further, Cajal bodies are the site where scaRNAs (small Cajal body RNAs) direct the pseudouridylation and 2'-*O*-methylation of spliceosomal snRNAs (33, 34). hTR may undergo similar modifications within Cajal bodies, although no such modifications have yet been identified for hTR.

The results presented here demonstrate that hTERT expression is necessary and sufficient for localization of hTR to Cajal bodies, supporting a role for this nuclear structure in the assembly of the telomerase RNP (Figs. 3 and 4 and see [6]). To date, we have not been able to detect a significant amount of the endogenous hTERT protein within Cajal bodies; however, we have seen hTERT residing within Cajal body adjacent structures during S phase of the cell cycle (7). We also cannot rule out that there is a small amount of hTERT within Cajal bodies that escapes detection using our IF conditions. In this work, we show exogenously expressed hTERT localizes to Cajal bodies in a manner influenced by the level of hTR present (Figure 3.8 but see also [6]), further implicating Cajal bodies as the site where active telomerase is produced from its

constituent components. Also, the increase in expression of both hTR and hTERT leads to a significant increase in the number of Cajal bodies present in each nucleus (Figure 3.8, compare doubly transfected cells to empty vector controls). This rise in the number of Cajal bodies is likely a response to an accelerated need for telomerase assembly; the high levels of exogenous hTERT and hTR could overwhelm the standard number of Cajal bodies and induce the formation of more. The assembly of hTR and hTERT into an active telomerase enzyme may be mediated by the SMN (survival of motor neurons) complex, an RNP chaperone known to reside within (and in foci beside) Cajal bodies (35). In fact, SMN has been shown to interact with telomerase (36) as well as the telomerase-associated protein Gar1 (37).

Cajal bodies are mobile nuclear structures and have been shown to interact with several chromosomal loci (38, 39), including telomeres (9). Recent evidence has implicated Cajal bodies in the delivery of telomerase to the telomere in cancer cell lines (7, 9). While we did not test this hypothesis directly, our data supports this notion. The coilin staining patterns observed in cells ectopically expressing hTR and hTERT closely resemble the staining pattern exhibited by telomere binding proteins (such as TRF2). The predominant hTERT foci exhibited in these cells exhibit a one-to-one colocalization with TRF2 (Figure 3.7A) and coilin (Figure 3.8), suggesting that Cajal bodies containing hTERT are docked at the telomere (i.e. there are hTERT, coilin, and TRF2 triple colocalizations). Taken together, our data fits with the model that telomerase is constructed within Cajal bodies, which then transport their assembled cargo to telomeres to carry out its function (telomere elongation).

In this study, we uncovered that the key components of telomerase, hTR and hTERT, influence the intranuclear trafficking of each other. Future work will be aimed at uncovering other aspects of regulation, including how the cell cycle controls the localization of telomerase to telomeres.

Materials and Methods

Cell culture and transfection

HeLa, VA-13, VA-13-hTR, GM847 and GM847-hTERT cells were grown on coverslips in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (FCS) (Mediatech). IIICF-T/C3 cells were grown in RPMI (American Type Culture Collection (ATCC), Manassas, VA) supplemented with 10 % FCS. IMR90 and IMR90-hTERT primary fibroblasts were grown in Minimum Essential Eagle's media (ATCC) with 10% FCS. MCF7 cells were cultured in Minimum essential medium Eagle's media supplemented with 0.01 mg/mL bovine insulin (Sigma-Aldrich, St. Louis, MO) and 10% FCS. All HME cells were grown in MEGM media supplemented with bovine pituitary extract (BPE) (Cambrex, Walkersville, MD). All cells were cultured at 37°C with 5% CO_2. Transfections with hTR and hTERT expressing plasmids and empty vectors (in pBluescript II SK+ and pcDNA6, respectively (25); vectors co-transfected with a mass ratio of 1:5 (Figure 3.7) or 1:2.5 (Figure 3.8)) were performed using the Lipofectamine 2000 reagent according to the manufacturer instructions (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, cells were plated on glass coverslips and processed for hTERT immunostaining 24 hours later (see below). All other transfections were carried out using Fugene transfection reagent, according to the manufacturers protocol (Roche, Indianapolis, IN). For hTERT RNAi, cells were

transfected with a shRNA against hTERT or empty vector control plasmid (21). 24 hours post transfection, cells were selected with 1 μg/mL puromycin for 48 hours prior to analysis.

hTR FISH

Probes complimentary to different regions of telomerase RNA were synthesized by Qiagen (Valencia, CA) as follows: hTR 128-183 (probe 1), GCT*GACATTTTT*TGTTTGCTCT*AGAATGAACGGT*GGAAGGCGGCAGGCCGAGGCT*T; hTR 331-383 (probe 2), CT*CCGTTCCTCTTCCT*GCGGCCTGAAAGGCCT*GAACCTCGCCCT*CGCCCCCGAGT*G; hTR 393-449 (probe 3), AT*GTGTGAGCCGAGT*CCTGGGTGCACGT*CCCACAGCTCAGGGAAT*CGCGCCGCGCT*C (T* indicates aminoallyl-modified thymidines). Probes were conjugated with cy3 mono-functional reactive dye according to the manufacturers protocol (GE Healthcare, Little Chalfront, Buckinghamshire, United Kingdom). 25 ng of each cy3 labeled hTR FISH probe were used per coverslip. hTR FISH was performed essentially as described (6, 7), http://www.singerlab.org/protocols). Cells were grown on coverslips overnight and then washed one time with 1X phosphate-buffered saline (PBS) (137 mM NaCl, 10mM Na_2HPO_4, 2.7 mM KCl, and 1.4 mM KH_2PO_4, pH7.4) and fixed with 4% formaldehyde (Electron Microscope Sciences, Fort Washington, PA), 10% acetic acid, and 1X PBS for 10 minutes at room temperature. After 2 PBS washes, cells were permeablized in 70% ethanol overnight at 4°C. When hTR FISH was combined with visualization of GFP, cells were fixed in 4% formaldehyde in 1X PBS for 10 minutes at room temperature. Following two PBS washes, cells were permeablized in 0.2% Triton

X-100 (Sigma-Aldrich) in 1X PBS for 5 minutes at 4°C. Cells were rinsed twice in 1X PBS and once in 50% formamide (Sigma-Aldrich), 2X SSC prior to FISH.

Coilin and TRF1 Immunofluorescence (IF)

Following FISH, cells were washed three times with 1X PBS. Next, cells were incubated with either one or both of the following primary antibodies at the indicated dilution for 1 hour at room temperature: mouse anti-p80 coilin (1:10,000; gift from G. Matera, Case Western Reserve University, Cleveland, OH), mouse anti-TRF2 (1:1000, Upstate/Millipore, Billerica, MA) and rabbit anti-TRF1 (1:100, gift from Susan Smith, Skirball Institute, New York, NY). Cells were washed three times in 1X PBS and then incubated with secondary antibody (1:100 Cy2 conjugated goat anti-rabbit IgG (H+L), 1:100 cy2 conjugated goat anti-mouse IgG (H+L), or 1:100 Cy5 conjugated goat anti-mouse IgGγ; all secondary antibodies obtained from Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. All antibodies were diluted in 0.05% Tween-20 in PBS (PBST). Cells were then subjected to three final 1X PBS washes and mounted in either 90% glycerol, 1 mg/mL ρ-Phenylenediamine, 1X PBS, and 0.1 μg / mL 4'6-diamidino-2-phenylindole (DAPI) or Prolong Gold (Molecular Probes/Invitrogen, Carlsbad, CA).

Cajal body induction

To induce Cajal body formation in primary cells, IMR90 and HME cells were cultured at 32°C for 24 hours prior to fixation as described (17).

5-Bromodeoxyuridine (BrdU) Labeling

BrdU labeling was performed essentially as described (7). Prior to fixation, cells were incubated with 100μM BrdU (Sigma-Aldrich) for 30 minutes at 37°C. The cells were

fixed as described above and denatured in 70% formamide (Sigma-Aldrich), and 2X SSC for 5 minutes at 80°C. Following 3 PBS washes, BrdU was detected using a monoclonal antibody (1:1000, G3G4; Developmental Studies Hybridoma Bank, Iowa City, IA) and AMCA-conjugated secondary antibody (1:100; Jackson ImmunoResearch) for 2 hrs at room temperature. Both antibodies were diluted in PBST. After three PBS washes, coverslips were mounted as described above. If hTR FISH was to be performed, cells were fixed again in 4% formaldehyde in 1X PBS for 10 minutes at room temperature and washed twice in PBS.

S phase synchronization

Synchronous populations of IMR90 and IMR90-TERT cells were obtained by double thymidine block. Cells were treated with 2mM thymidine (Sigma-Aldrich) for 36 hours. Cells were released by rinsing twice with 1X PBS and replacing the normal growth media for 12 hours. Cells were retreated with 2mM thymidine for another 36 hours. At various time points after release, cells were fixed and analyzed by BRDU labeling and FISH.

hTERT IF

When the 2C4 antibody was used (Figure 3.4), hTERT IF was performed essentially as described (7, 21). Cells were grown on coverslips, rinsed once in 1X PBS and fixed in chilled acetone for 5 minutes. Cells were washed twice in 1X PBS then incubated with 2M HCl for 20 minutes at room temperature. Cells were again rinsed in 1X PBS and neutralized in 0.1M boric acid, pH 8.5 for 10 minutes at room temperature. Cells were blocked overnight in 1% BSA in 1X PBS at 4°C, then incubated with 1:5000 dilution of 2C4 monoclonal hTERT antibody (Abcam, Cambridge, MA) for 1 hour at room

temperature. Following 3 1X PBS washes, cells were incubated with a 1:100 dilution of cy2 conjugated goat anti-mouse IgM secondary antibody for 1h at room temperature. When combined with coilin IF, the mouse anti-coilin antibody described above and a cy3 conjugated goat anti-mouse IgGγ were used. All secondary antibodies were obtained from Jackson ImmunoResearch.

When the rabbit polyclonal hTERT antibody (600-401-252, Rockland Immunochemicals, Gilbertsville, PA) was used (Figures 3.7 and 3.8), hTERT IF was performed under the following conditions: Cells were plated on coverslips, then incubated with CSK buffer (0.5% Triton X-100, 20mM HEPES-KOH, pH 7.9, 50mM NaCl, 3mM $MgCl_2$, 300mM sucrose) for 7 minutes at 4°C to extract soluble proteins. The cells were immediately fixed with 4% formaldehyde in 1X PBS for 10 minutes at room temperature. The cells were blocked in a solution of 5% milk, 0.05% Tween-20 in 1X PBS for 1 hour at room temperature. Cells were successively incubated for 1 hour at room temperature with 1:600 dilution of the rabbit anti-hTERT antibody and either a 1:200 dilution of monoclonal anti-TRF2 antibody (clone 4A794, Upstate/Millipore) or a 1:1000 dilution of monoclonal anti-coilin antibody (ab11822, Abcam); all antibodies diluted in blocking solution. Slides were then washed with 0.05% Tween-20 in PBS 1X 3 x 5 min at room temperature. To detect primary antibodies, cells were incubated for 40 min at room temperature with cy3- or Alexa 488-conjugated donkey secondary antibodies (Jackson Immunoresearch Laboratories and Molecular Probes, respectively) diluted 1:400 in blocking solution. DNA was counter-stained with 100 ng/ml DAPI in 1X PBS.

Microscopy

In most experiments, images were obtained using a Zeiss Axioskop 2 Mot Plus fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were acquired at 63x (Plan Apochromat objectives, numerical aperture 1.4) using a cooled charge-coupled device Retiga Exi Fast 1394 camera (Qimaging, Burnaby, British Columbia, Canada) and IPLab Spectrum software. For Figures 3.7 and 3.8, images were acquired using the LMS510 confocal microscope (Zeiss) equipped with UV (wavelength 375 nm), Argon (wavelength 457/488/514), and HeNe (wavelength 543) lasers. The LMS510 (Zeiss) and Adobe Photoshop software were used for image analysis.

RNAse Protection Analysis (RPA)

Total RNA samples were prepared from cells using Trizol according to the manufacturers protocol (Invitrogen, Carlsbad, CA). RNAse A/T1 protection was carried out as described (40). Antisense U3 and U1 probes were synthesized from *Eco*RI linearized plasmids (41, 42) using SP6 polymerase. The template for the hTR probe was generated by PCR amplification of an hTR plasmid (43) using the following oligos: AGCCGCGAGAGTCAGCTTGG (5'end) and ATTTAGGTGACACTATAGAGGTGACGGATGCGCACGATC (3' end). The antisense hTR probe was generated by in vitro transcription of this PCR product using SP6 polymerase.

Chromatin Immunoprecipitation (ChIP)

$6.5x10^6$ HEK293T cells were seeded in 60 mm dishes and transfected the same day with 1.6 µg and 6.4 µg of hTERT- and hTR-encoding plasmids, respectively

(or the empty vector controls) using Lipofectamine 2000 according to the manufacturer's intruction (Invitrogen). The next day, cells were trypsinized and transfered to 100 mm dishes. Fourty-eight hours post-transfection, cells were collected for the ChIP experiment. Methods for ChIP were previously described (25). The equivalent of $2x10^6$ cells was used in each immunoprecipitation. For immunoprecipitations, 5 µg of TRF1(N19, Santa-Cruz) or 25 µl of hTERT (R484, crude serum).

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Figure 3.1. Localization of hTR to intranuclear foci is not affected by hTR levels or Cajal body formation *A. Ectopic overexpression of hTR does not lead to localization of hTR to nuclear foci.* IMR90 primary cells transiently transfected with an hTR construct (14), VA13, and VA13 cells stably expressing the same construct were analyzed by hTR FISH. GFP marks cells transfected with the hTR construct. Nuclei are stained with DAPI. *B. Comparison of hTR levels amongst normal, ALT and HeLa cells.* RNAse protection assay on total RNA from HeLa, IIICF-T/C3, GM847, IMR90, VA13, and VA13-hTR cells. U3 snoRNA and U1 snRNA were used as loading controls. *C. Induction of Cajal body formation does not affect hTR localization.* MCF7 breast cancer cells and HME primary cells were grown at 37°C (normal growth temperature) or 32°C (to induce Cajal body formation). Cells were costained by hTR FISH (hTR panels) and coilin IF (marker protein for Cajal bodies, coilin panels). Arrowheads denote Cajal bodies that contain hTR. Open arrowheads indicate Cajal bodies without hTR. *D. hTR does not localize to Cajal bodies in the ALT cell lines GM847 and IIICF-T/C3.* HeLa, GM847, and IIICF-T/C3 cells were co-analyzed by hTR (detected by FISH, hTR panels) and coilin (detected by IF, coilin panels). Arrowheads denote Cajal bodies. DIC, differential interference contrast. Scale bars, 10 μm.

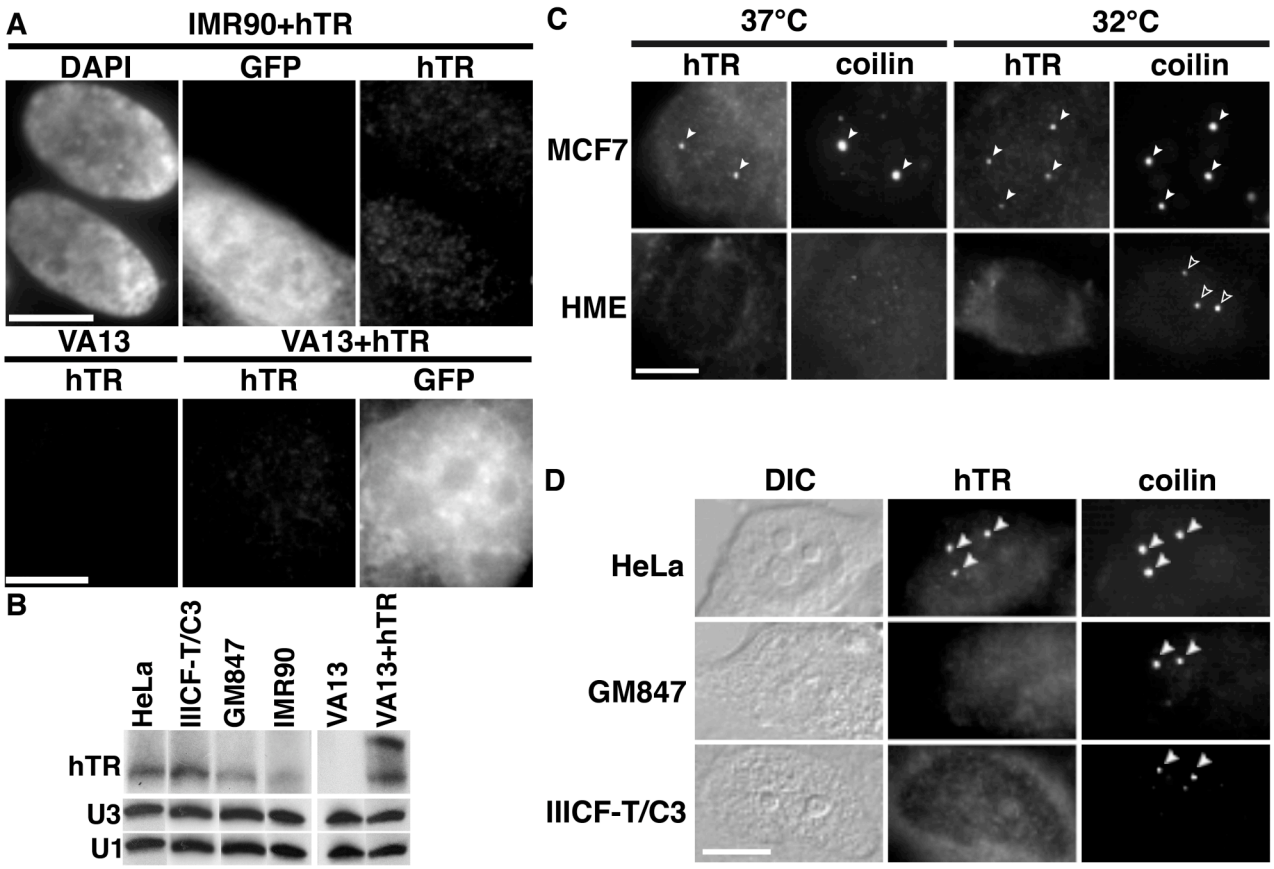

A
IMR90+hTR
DAPI
GFP
hTR
VA13
VA13+hTR
hTR
hTR
GFP
B
HeLa
IIICF-T/C3
GM847
IMR90
VA13
VA13+hTR
hTR
U3
U1
C
37°C
32°C
hTR
coilin
hTR
coilin
MCF7
HME
D
DIC
hTR
coilin
HeLa
GM847
IIICF-T/C3

Figure 3.2. hTERT expression leads to accumulation of hTR within nuclear foci. MCF7 breast cancer cells, HME normal cells, and HME cells expressing some combination of hTERT, large T, and oncogenic (onco.) Ras were subjected to hTR FISH (hTR panels). Nuclei are stained with DAPI. Arrowheads denote hTR foci. Scale bar, 10 μm.

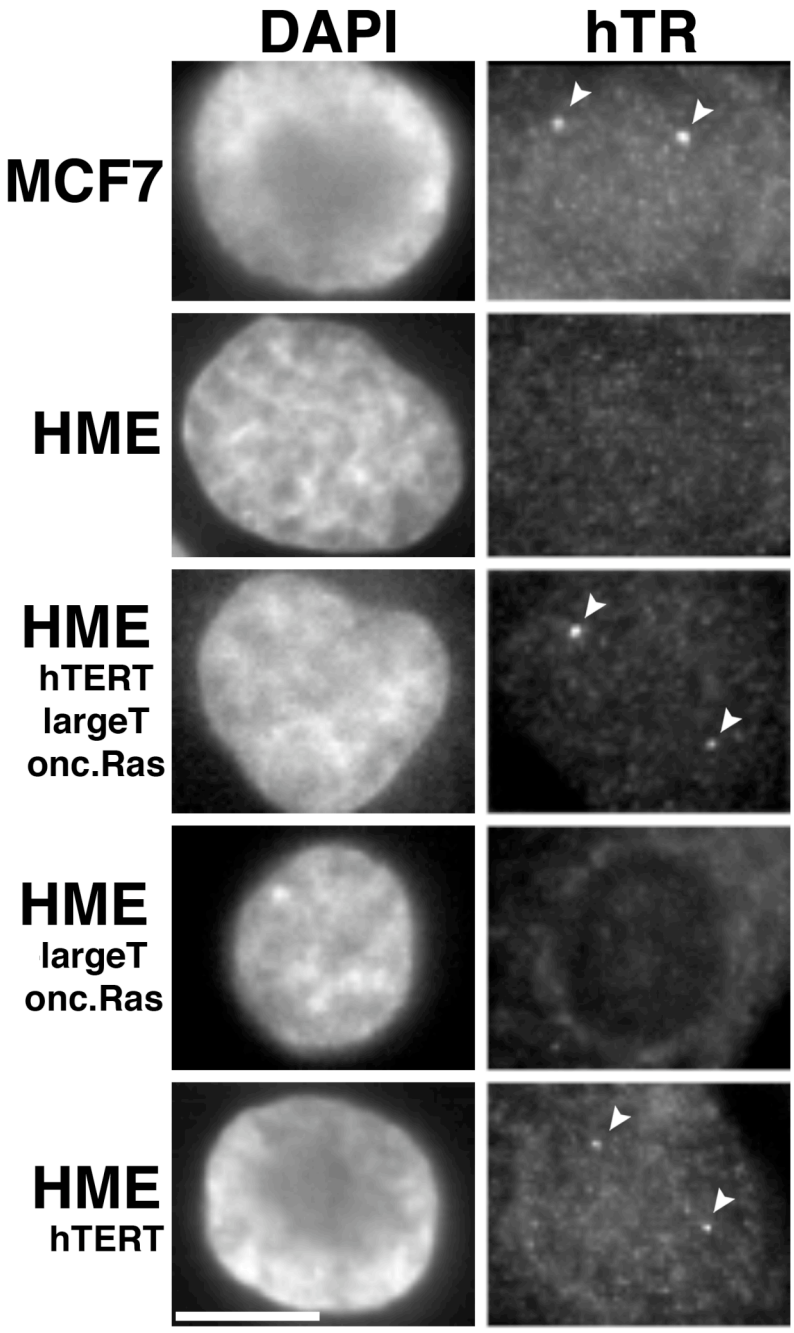

DAPI
hTR
MCF7
HME
HME
hTERT
largeT
onc.Ras
HME
largeT
onc.Ras
HME
hTERT

Figure 3.3. Expression of hTERT induces hTR localization to Cajal bodies. HME cells that ectopically express hTERT were cultured at 32˚C to induce Cajal body formation. These cells and GM847 cells that stably express hTERT were analyzed by hTR FISH (hTR panels) and coilin IF (coilin panels). Arrowheads indicate hTR foci that colocalize with coilin (Cajal bodies). Scale bars, 10 μm.

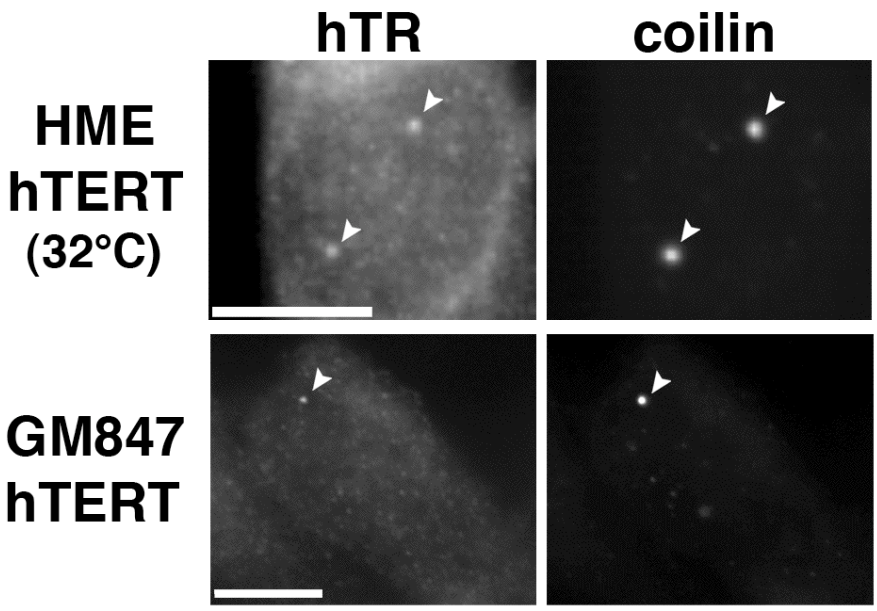

hTR
coilin
HME
hTERT
(32°C)
GM847
hTERT

Figure 3.4. hTERT expression is necessary for localization of hTR to Cajal bodies. *A. Knocking down hTERT leads to loss of hTR from Cajal bodies.* HeLa cells were transfected with hTERT shRNA (2nd and 3rd rows) or an empty vector control (top row). Cells were co-analyzed by hTERT (detected with the 2C4 monoclonal antibody) and coilin IF in the left set of panels and hTR (detected by FISH) and coilin (detected by IF) in the right set of panels. Data in each set of panels were normalized relative to the empty vector control to allow direct visual comparison. Arrowheads indicate hTR in Cajal bodies. Open arrowhead indicates Cajal body with reduced level of hTR. DAPI was used to stain the DNA. Scale bar, 10 μm. *B. Knocking down hTERT does not affect hTR levels*. RNAse protection assay on total RNA from HeLa cells transfected with hTERT shRNA or empty vector. U3 snoRNA and U1 snRNA are used as loading controls.

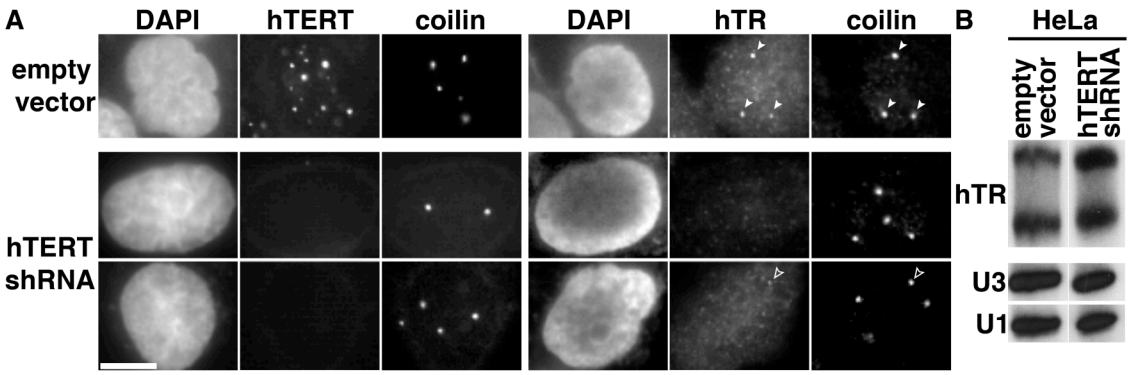

A
DAPI
hTERT
coilin
DAPI
hTR
coilin
B
HeLa
empty vector
hTERT shRNA
empty vector
hTERT shRNA
hTR
U3
U1

Figure 3.5. Localization of hTR in nuclear foci is S phase dependent. *A. hTR foci are predominantly found in S phase cells in normal cells that express hTERT.* IMR90 and IMR90-hTERT cells were subjected to hTR FISH (hTR panels) and BrdU labeling (BRDU panels). Arrowheads indicate hTR foci, which are present in BrdU-positive IMR90-TERT cells. Note that the BrdU-negative IMR90-hTERT cell in the middle set of panels does not contain any hTR foci. DIC, differential interference contrast. Scale bar, 10 μm. *B. Synchronization of IMR90-TERT in S phase increases the percentage of cells with hTR foci*. The percentage of IMR90-TERT cells with hTR foci is graphed relative to time after release from a double thymidine block. Data collected from two independent experiments. A, asynchronous cells.

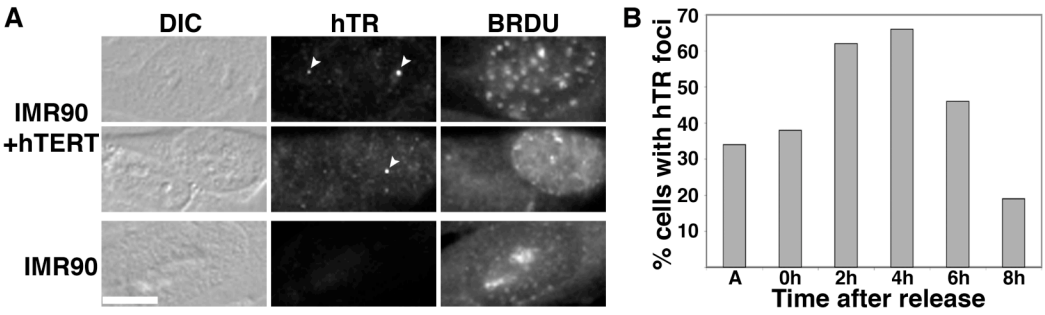

A
DIC
hTR
BRDU
IMR90
+hTERT
IMR90
B
% cells with hTR foci
70
60
50
40
30
20
10
A
0h
2h
4h
6h
8h
Time after release

Figure 3.6. hTERT expression is sufficient to localize hTR to telomeres. *A. hTR localizes to a subset of telomeres in normal cells that ectopically express hTERT.* IMR90-hTERT (top panels) and HME-hTERT cells were analyzed by hTR FISH (hTR, red) and TRF1 IF (TRF1, green). Merge panels show superimposition of hTR and TRF1 panels; yellow indicates an overlap of signal. Arrowheads denote colocalization of hTR and telomeres. *B. hTR localizes to a majority of telomeres in VA13-hTR+hTERT cells.* hTR FISH (hTR, red) and TRF2 IF (TRF2, green) were performed on VA13 cells that stably express both hTR and hTERT. Arrowheads point to representative examples of hTR and TRF2 colocalizations in each panel. Scale bars, 10 μm.

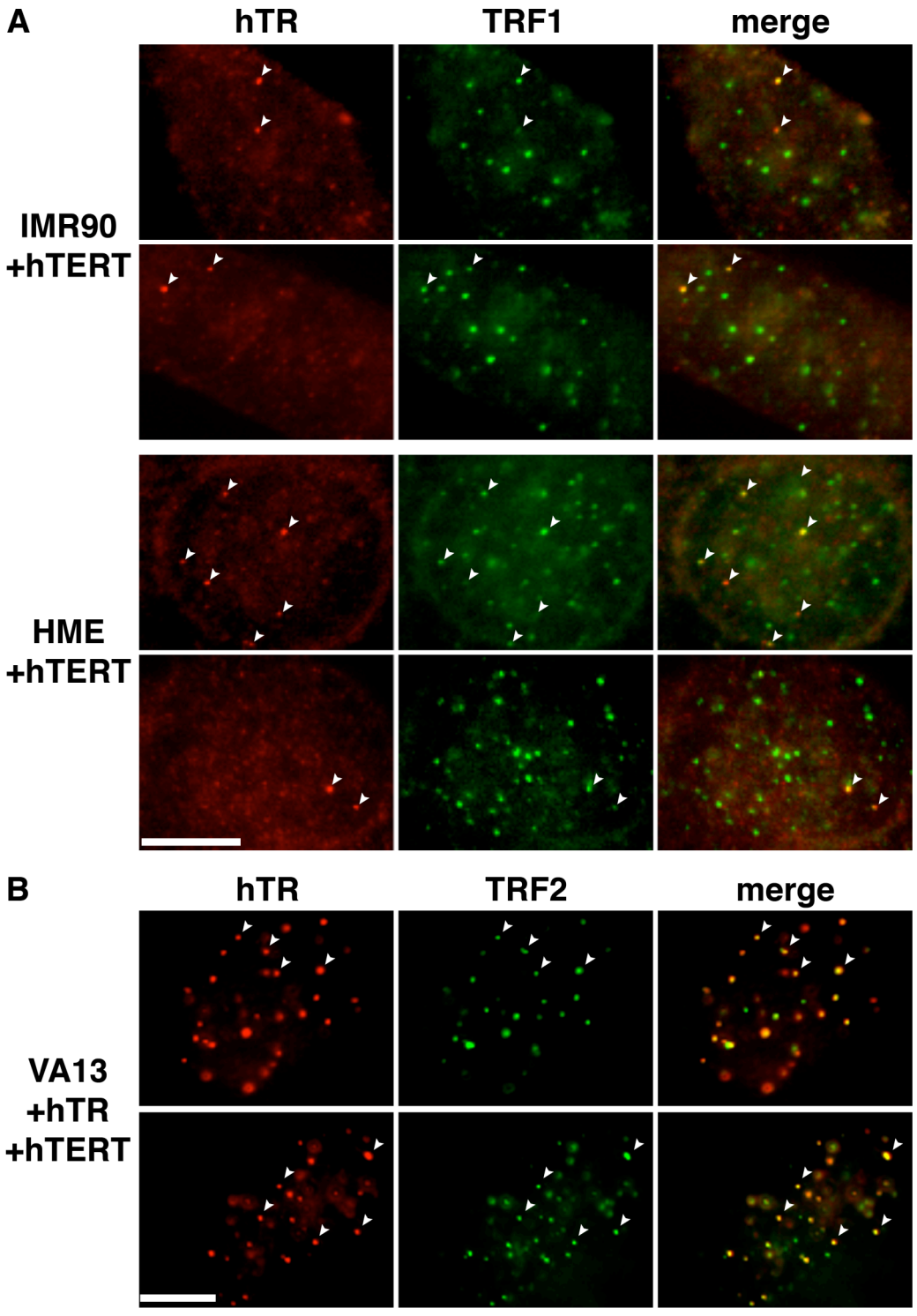

A
hTR
TRF1
merge
IMR90
+hTERT
HME
+hTERT
B
hTR
TRF2
merge
VA13
+hTR
+hTERT

Figure 3.7. hTR expression enhances association of hTERT with the telomere. *A. The number of hTERT foci colocalizing with telomeres increases upon expression of hTR.* hTERT (detected by IF using a rabbit polyclonal antibody, green) and TRF2 (detected by IF, red) staining on HeLa cells transiently transfected with vectors encoding hTR and/or hTERT (+) or empty vectors (-) as indicated. Arrowheads in merge panels point to hTERT foci at telomeres. *B. The overall amount of hTERT at the telomeres increases upon expression of both hTR and hTERT.* ChIP analysis on the transiently transfected HeLa cells. Immunoprecipitations were performed with either TRF1 or hTERT antibodies as indicated, the beads only served as a negative control. The bar graph indicates a quantitation of the amount telomeric DNA pulled down with the hTERT antibodies for each transfection condition. *C. hTERT is expressed in cells transfected with the hTERT vector.* Western blot using the polyclonal antibody against hTERT on the transiently transfected cells. Tubulin is used as a loading control.

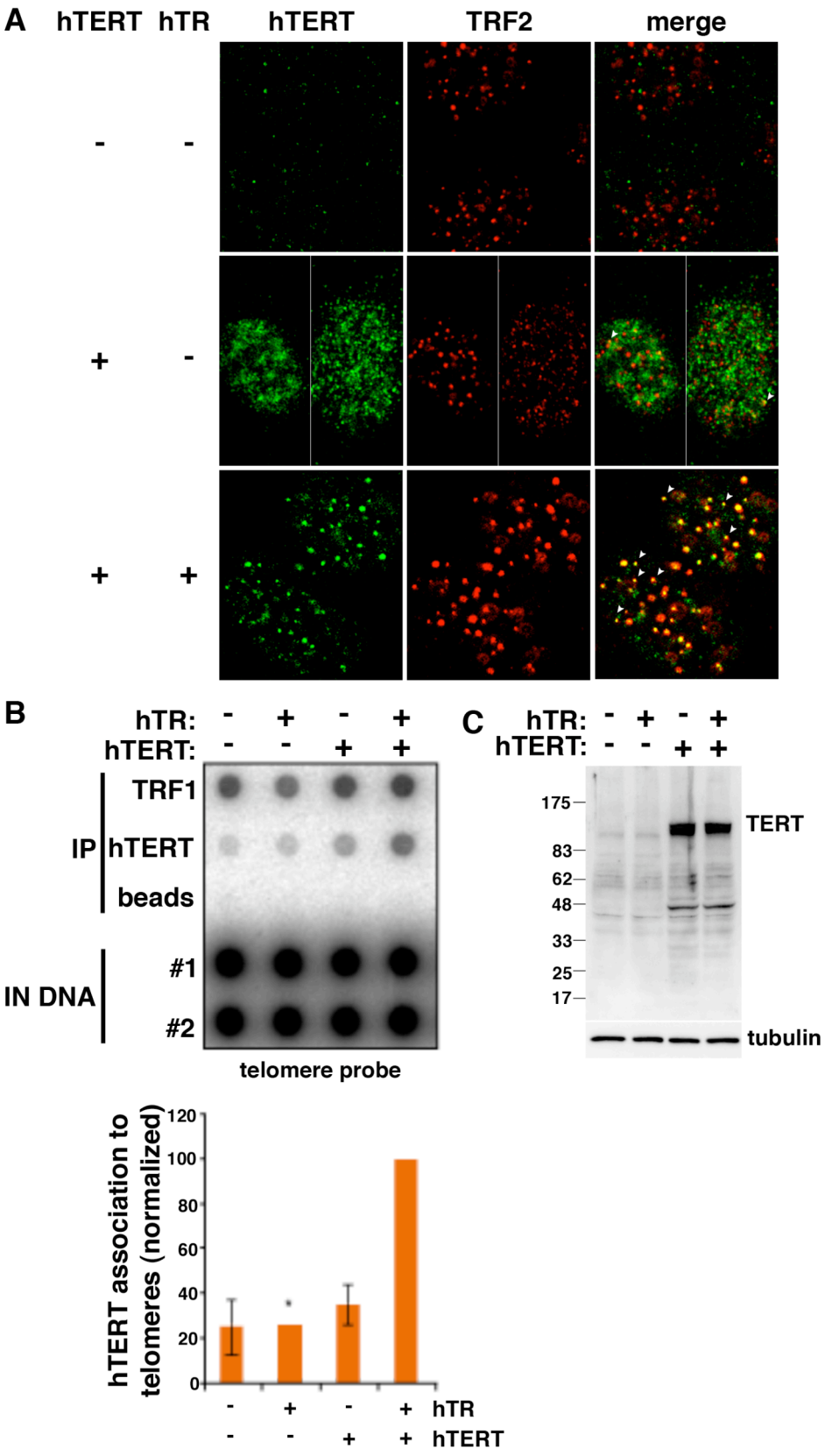
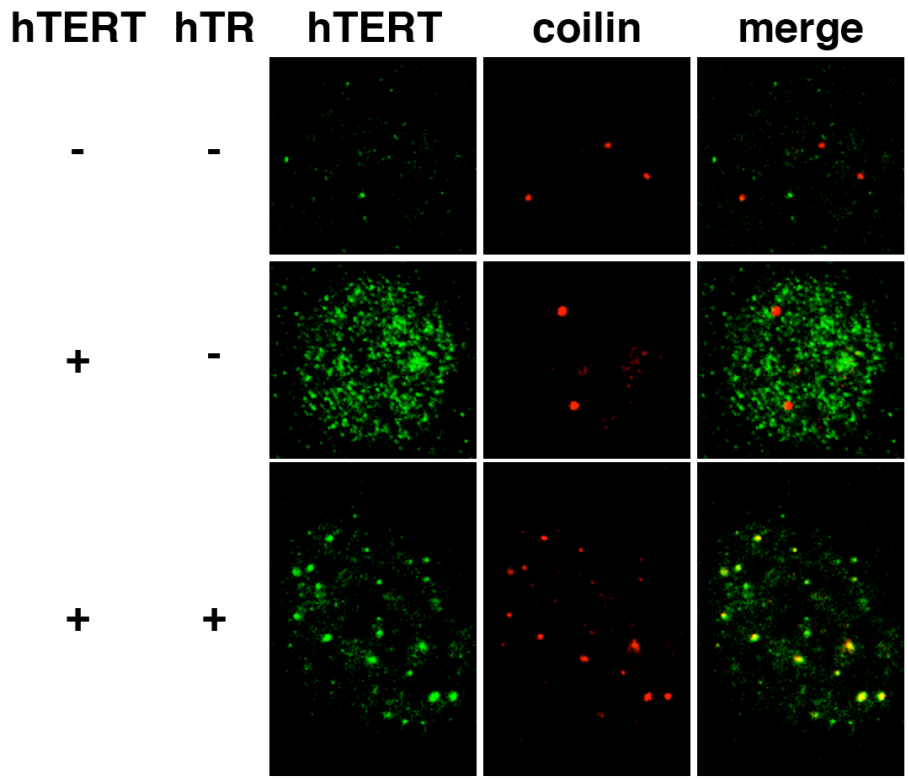

A
hTERT
hTR
hTERT
TRF2
merge
B
hTR:
hTERT:
TRF1
IP
hTERT
beads
IN DNA
#1
#2
telomere probe
C
TERT
tubulin
175
83
62
48
33
25
17
hTERT association to telomeres (normalized)
120
100
80
60
40
20
0
hTR
hTERT

Figure 3.8. Expression of hTR increases the detection of hTERT within Cajal bodies. hTERT (green, detected with a rabbit polyclonal antibody) and coilin (red) IF were performed on HeLa cells transiently transfected with vectors encoding hTR and/or hTERT (+) or empty vectors (-) as indicated. Yellow in merge panels indicates an overlap of hTERT and coilin signal.


hTERT
hTR
hTERT
coilin
merge
-
-
+
-
+
+

Chapter 4

Mouse Telomerase RNA Localizes to Telomeres During S Phase Independent of Cajal Bodies[1]

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[1]Rebecca L. Tomlinson, Bradley R. Culp, A. Gregory Matera, Rebecca M. Terns and Michael P. Terns, to be submitted to *Molecular Biology of the Cell*

Abstract

The intranuclear trafficking of human telomerase involves a dynamic interplay between multiple nuclear sites, most notably Cajal bodies and telomeres. Cajal bodies are thought to serve as sites of telomerase maturation, storage, and assembly, as well as to function in the delivery of telomerase to telomeres, the site of telomerase action. Here, we find that mouse telomerase RNA (mTR) does not localize to Cajal bodies, and instead resides in separate nuclear foci throughout much of the cell cycle. A portion of these foci localize to subsets of telomeres specifically during S phase. The localization of mTR to telomeres does not require Cajal bodies, as cells derived from coilin knockout mice display mTR at telomeres at frequencies similar to their wild-type counterparts. Instead, mice seem to employ another method for telomerase delivery to telomeres. mTR foci are grouped together into chains specifically during S phase with at least one of the mTR foci within each chain colocalizing with a telomere. The appearance of these chains coincides with the timing observed for telomere localization, raising the possibility that these chains may serve in some capacity to transport mTR to the telomere.

Introduction

Telomeres are capping structures that comprise the physical ends of eukaryotic chromosomes. In vertebrates, they consist of tandemly repeated arrays of TTAGGG and a number of associated proteins (1). Telomeres serve a protective function, first preventing the ends of chromosomes from being recognized as double strand breaks, blocking illegitimate recombination events and breakage-fusion-bridge cycles (reviewed

in [2]). Second, telomeres serve as a barrier to the loss of genetic information due to the inability of DNA polymerases to fully replicate the ends of linear DNAs (the so-called end replication problem) (3). This means a portion of the telomere is lost with each cell division. Eventually, this telomere attrition triggers the cell to stop dividing and enter into a state of proliferative senescence (4, 5).

Telomerase is the specialized reverse transcriptase that combats this telomeric erosion by adding additional repeats to the 3' end of telomeric DNA. Telomerase is comprised of two essential components: telomerase RNA (TR), which provides the template for repeat addition and telomerase reverse transcriptase (TERT), which synthesizes the repeats (6-9). Telomerase activity is not detected in most human somatic cells, but the enzyme is active in the vast majority (over 90%) of human cancers and is responsible for the prolonged proliferative capacity of these cells (10-12). Insights into the biogenesis and regulation of this enzyme thus have potential anti-cancer applications.

Localization studies by our lab and others using human cell lines (13-16) have begun to elucidate the trafficking pathway of the telomerase RNP. Using fluorescence *in situ* hybridization (FISH), we determined that human TR is detected throughout interphase within Cajal bodies (13-16), dynamic nuclear structures that have been implicated in the biogenesis of several cellular ribonucleoproteins (RNPs) (reviewed in [17-19]). This localization occurs in telomerase-positive cancer cells but not telomerase-negative normal cells (16), indicating that Cajal bodies could serve as sites of telomerase assembly. Detection of human TERT (by immunofluorescence, IF) revealed the protein is present in distinct nucleoplasmic foci, separate from Cajal bodies

(15), but evidence exists that exogenously expressed hTERT does reside within Cajal bodies ([16] and see Chapter 3). During S phase, both human TR and TERT exhibit a dynamic shift in location, first associating with nucleoli before localizing to foci adjacent to Cajal bodies (15). Throughout S phase (but peaking in mid-S), both hTR and hTERT associate with specific subsets of telomeres (14, 15), corresponding to the time of telomere replication (and possible synthesis) in these cells (20, 21). The timing of localization to telomeres corresponds to emergence of the Cajal body adjacent foci (15). This, taken together with the fact that Cajal bodies (containing hTR) transiently associate with subsets of telomeres during S phase (14), indicates that Cajal bodies may act to deliver telomerase to the telomere.

Recently we discovered that localization of human TR to both Cajal bodies and telomeres is dependent upon TERT expression (Chapter 3), but other factors that influence the trafficking of telomerase remain unknown. To gain a better understanding of regulation of telomerase trafficking, we have turned our attention to the mouse model system. The genetic tools available for mice have contributed to our understanding of basic telomere and telomerase biology, particularly with regards to the roles they play in cancer and aging (reviewed in [22, 23]). Here, we have developed a FISH procedure specific for detection of mouse telomerase RNA (mTR) and used this technique to characterize the localization patterns of mTR in cultured mouse cell lines. Unlike human TR, mTR does not colocalize with coilin or any other Cajal body marker (such as the survival of motor neurons protein (SMN), or U85 small Cajal body (sca)RNA), and is instead found in separate nuclear foci. A portion of the mTR foci do localize to telomeres selectively during S phase of the cell cycle. Also during S phase a linking of

individual mTR foci into chains of connected spheres is observed. At least one of the foci in each chain will often correspond to a telomere. Furthermore, the occurrence of these chains mimics the timing for telomere localization, suggesting that this may be the method employed for delivery of mTR to the telomere.

Results

mTR FISH procedure is specific for the detection of mouse telomerase RNA

Here, we have established a FISH procedure specific for the detection of mouse telomerase RNA (mTR) in order to examine its subcellular distribution. A total of four probes were designed against different regions in mTR; the regions encompassed by each probe are highlighted in Figure 4.1A (see Materials and Methods for probe sequences). The two probes used throughout this work are denoted by asterisks. Upon hybridization with these two probes (probe 1 and probe 2), 1-3 small, spherical foci were detected in the nuclei of cultured mouse embryonic fibroblast (MEF) cells (Figure 4.1B); approximately 2/3 of the cells in a given population of MEF cells displayed these foci. Hybridization with either probe alone, or any of the other two remaining probes gave a similar localization pattern, and maximal signal is seen when two probes were used in combination (data not shown).

Several controls demonstrated the specificity of this procedure for detection of mTR. First, mTR signal was lost upon treatment of the cells with RNAse A prior to hybridization with our two mTR probes (Figure 4.1B). As controls, we also probed the RNAse treated cells with probes against U3 snoRNA (positive control for treatment) and the telomeric repeats (negative control for treatment). As expected, the U3 signals were

eliminated or severely reduced in the RNAse treated cells, while telomeric signals were virtually unaffected by the treatment (data not shown). Furthermore, no foci were observed when MEF cells derived from mTR knockout mice were examined by the FISH procedure (Figure 4.1B). Also, we used the mTR FISH probes in HeLa (human cervical carcinoma) cells, and detected no foci (data not shown), further indicating the specificity of our procedure for detection of mTR.

mTR does not localize to Cajal bodies in cultured mouse cells

Human TR localizes to intranuclear Cajal bodies throughout interphase in telomerase-positive human cancer cell lines (13, 16). To see if any of the mTR foci observed in the MEF cells would correspond to Cajal bodies, we combined our mTR FISH procedure with immunofluorescence (IF) using antibodies directed against coilin, the hallmark protein of Cajal bodies (24). Surprisingly, none of the mTR foci present in the MEF cells colocalized with coilin (Figure 4.2, top set of panels). We next examined a range of different mouse cell lines representing different tissue sources, and they all showed the same phenotype, mTR in foci separate from Cajal bodies (Figure 4.2). To date, we have only detected mTR colocalizing with coilin in one mouse cell line (A9 myeloma cells) (data not shown).

We subsequently examined a group of different markers for Cajal bodies to see if they overlapped with any of the mTR foci. U85, a small Cajal body (sca)RNA and SMN (the survival of motor neurons protein) are other prototypical markers for Cajal bodies, whereas Nopp140 localizes to both Cajal bodies and nucleoli in the nuclei of mammalian cells (25-27). When coilin expression is eliminated, these markers still accumulate into separate nucleoplasmic foci known as residual Cajal bodies (28, 29),

suggesting these markers can exist in nuclear foci independent of coilin (and thus may overlap with our mTR foci). In our MEF cell line, no colocalization between mTR and any of these markers was observed (Figure 4.3), further indicating that mTR does not reside within Cajal bodies in mouse cell lines. We also examined whether mTR would colocalize with the snoRNP protein fibrillarin and the U3 snoRNA (markers that display the same localization pattern as Nopp140), but found no instances of signal overlap (data not shown). Finally, we looked at mTR localization in MEF cells derived from coilin knockout mice (29)(Figure 4.3B). mTR foci were observed at the same frequency in coilin knockout MEFs as their wild-type counterpart. Within the coilin knockout MEFs, the mTR signals also did not overlap with any of the residual Cajal body markers described above (data not shown). Taken together, our data suggests mTR does not localize to Cajal bodies and is instead housed in its own separate nuclear structure.

mTR is found at a subset of telomeres in S phase cells

Given that mTR does not localize to Cajal bodies in cultured mouse cells, we set about to determine if any of the mTR foci localized to telomeres. When mTR FISH was combined with IF using antibodies against the telomere binding proteins TRF1, TIN2, or TPP1 or FISH using a probe directed against the telomeric repeats, we found that mTR colocalized with a subset of telomeres in around 10% of MEF and 3T3 cells that display mTR foci (Figure 4.4 and data not shown). This is similar to the percentages we previously observed for TR-telomere associations in human cancer cell lines, where human TR is found at a subset of telomeres specifically in S phase cells (14, 15). In order to see if this was an S phase-specific event in the mouse cells, we first stained the MEF and 3T3 cells with antibodies against PCNA (proliferating cell nuclear antigen).

PCNA is expressed by all interphase cells, but specifically associates with chromatin in cells undergoing DNA replication (i.e. cells in S phase of the cell cycle) (30). Using our fixation conditions, we were able to distinguish which cells were in S phase of the cell cycle and were also able to discern the S sub phase as early, mid, or late (Figure 4.4A). Upon staining with PCNA, we found that all mTR-telomere associations occurred solely in S phase cells (Figure 4.4A).

To further test the S phase-specificity of the localization of mTR to telomeres, we synchronized the MEF and 3T3 cells using a double thymidine cell cycle arrest. PCNA staining (verified in some instances by fluorescence activated cell sorting (FACS) analysis, data not shown) was performed to confirm synchronization efficiency of the cells. As can be seen in Figure 4.4B, the number of cells displaying mTR at the telomere gradually increased as cells progressed through S phase. Starting at G1/S (0h post release from the double thymidine block), 16% of cells with mTR foci showed at least one telomere association (18 of 113 cells with mTR foci). In early S (1 and 2h post release) and mid S (4h post release), the percentage increased steadily from 22% (1h data, 38 of 174 cells with mTR foci) to 28% (2h data, 55 of 196 cells with mTR foci) to 31% (4h data, 50 of 162 cells with mTR foci). The fraction of cells displaying mTR-telomere associations was highest in late S phase, where 38% of cells with mTR foci (6h data, 60 of 157 cells) had a direct overlap of mTR and telomere signals. These statistics represent data from two separate experiments; furthermore, the frequencies of mTR-telomere associations observed were similar when two different telomere markers (TIN2 and TPP1) were used. Our data clearly shows that the number of mTR foci

colocalizing with telomeres gradually increased over S phase, with more cells in late S displaying higher numbers of mTR-telomere associations per cell.

Recent reports have implicated Cajal bodies in the delivery of telomerase to telomeres in human cancer cells (14, 15). However, we did not see significant associations of mTR with Cajal bodies in cultured mouse cells (Figures 4.2 and 4.3), suggesting that Cajal bodies may not be necessary for delivery of mTR to the telomere. To further test the requirement for Cajal bodies in telomerase recruitment to telomeres in mouse cells, we performed mTR FISH and IF with the above telomere binding protein antibodies on the coilin KO MEF cells (Figure 4.5). We found that mTR localized to the telomere in the coilin KO MEFs (Figure 4.5). In fact, the percentage of mTR-telomere associations was the same between the wild type and coilin KO MEFs, with both cell lines displaying mTR at the telomere in roughly 12% of the cells in a given population.

Given that Cajal bodies are not playing a role in delivery of telomerase to telomeres in mouse cells, we wondered how mTR arrives at its functional site. During the course of our analysis with synchronized cells we noticed an interesting pattern of mTR localization: mTR foci linked together in chains of 2-5 foci. These mTR chains are similar to the nucleolar rings exhibited by human TR early in S phase of the cell cycle (15). To see if any of the chains were surrounding nucleoli, we co-stained the cells with U3 FISH probes and found less than 10% associated with nucleoli (Figure 4.6A), suggesting they were separate structures all together. However, for 60% of these chains, at least one of the mTR foci directly overlapped with a telomere (as marked by TIN2 or TPP1 staining) (Figure 4.6A). The appearance of the mTR chains coincided with the timing of mTR localizing to the telomere; MEF and 3T3 cells displayed a

gradual increase in mTR chains as cells progressed through S phase (Figure 4.6B and data not shown). Taken together, our data suggest that mTR foci may connect together into longer chains which function to ensure telomerase reaches its substrate telomere.

Discussion

Recent analyses of the trafficking of telomerase in human cells have garnered some insight into how the enzyme is regulated. Specifically, telomerase is recruited to subsets of telomeres only during S phase, providing a possible explanation as to how the enzyme is restricted to function only during this stage of the cell cycle. In this work, we describe the subcellular localization of mouse telomerase RNA and find key similarities and differences between the human and mouse systems. By extending our localization studies into mouse cells, we open up a new range of options to study factors that mediate the trafficking of telomerase.

mTR, like human TR, is found at distinct subsets of telomeres throughout S phase of the cell cycle (Figure 4.4A). Links of telomerase regulation to the cell cycle are not unusual for mouse cells; the Rb family of proteins has been shown to play a role in telomere elongation in this system (31). But why only certain mammalian telomeres recruit telomerase at a given time in the cell cycle remains a poorly understood process that may be a function of telomere length. Several lines of evidence suggest that telomerase exhibits a preference to extend the shortest telomeres in mouse cells (32, 33). When mice with short telomeres are crossed with those with long telomeres, the shorter telomeres are extended first in the resulting offspring (32). Likewise, when telomerase levels are limiting (in a mouse heterozygous for TERT expression), the

shortest telomeres in the population are maintained (while the longer ones grow shorter over time) (33). Further, molecular studies in yeast suggest that a small sampling of telomeres are extended by telomerase within any given cell cycle (34). This study also showed that telomerase exhibits a preference to act on the shortest telomeres, leading the authors to hypothesize that shorter telomeres are more prone to be in an "extendible", open state (34). In contrast, longer telomeres would then tend to be in a more closed, or "non-extendible" state (34). Taken with our data, this suggests that the subset of telomeres that recruit mTR (or human TR and TERT in human cancer cells) are more than likely in an extendible state (and thus represent the shorter telomeres in the population), although a direct correlation between telomere length and the frequency of elongation by telomerase has not been established for mammalian cells.

The association of mTR with telomeres gradually increases as cells progress through S phase (Figure 4.4B), differing from human cancer cells, where the association peaks during mid S (15). A recent study in yeast suggests that shorter telomeres are replicated earlier in S phase than average sized telomeres (35). The early replication of these telomeres coincides with telomere synthesis by telomerase (35). Perhaps since mouse telomeres on average exhibit much longer lengths than human telomeres (20-150 kb versus 5-15 kb for human cells) (36), they take longer to achieve an open ("extendible") chromatin state, and thus take longer to recruit telomerase. This possibility links DNA replication events to telomere synthesis (by telomerase) in mammalian cells, and further extends the possibility that shorter telomeres are extended preferentially by telomerase.

Contrary to what we observe for human cancer cells, mTR does not localize to Cajal bodies, and is instead found in distinct, nucleoplasmic foci throughout interphase (Figures 4.2 and 4.3). mTR retains an intact CAB box (with a sequence exactly matching that of the CAB box of human TR), a domain found in small Cajal body (sca)RNAs (and human TR) that functions to target and store the RNA within Cajal bodies, suggesting mTR should still transit through Cajal bodies (13, 27, 37). However, mTR does lack around 40 nucleotides of sequence found in the 5' domain of hTR upstream of the template region (37). Perhaps this sequence somehow functions in the localization to or retention of mTR within Cajal bodies. Another possibility exists, that telomerase foci and Cajal bodies are separate structures that are typically found coincident with one another in human cancer cells, but remain as solitary foci in the mouse cells we examined. This idea is not unusual, as SMN foci (also known as gems, short for gemini, or twins, of Cajal bodies [25]) are found separate from Cajal bodies in cells deficient in coilin methylation (38) and at certain times in development (39, 40). The cell lines we examined in this work (Figure 4.2) are derived from embryonic tissue (MEF, 3T3) cells, or remain in an undifferentiated state (n2a, c2c12), suggesting the mTR foci and Cajal body dissociation we observe could be a developmentally regulated phenomenon (similar to what has been observed between gems and Cajal bodies). A9, the anomalous cell line that displays mTR within Cajal bodies (data not shown), is an adult myeloma, adding further support to this notion. In fact, the Cajal body associated foci we observe in human cancer cells (15) could be reminiscent of these separate structures, indicating that the dissociation of foci could also be under cell cycle control. The possibility also exists that mTR does not enter Cajal bodies in most mouse cells,

and that this localization difference represents a fundamental dissimilarity between mouse and human telomerase biology.

Cajal bodies are proposed to have a role in transport of telomerase to the telomere in human cancer cells (14, 15). In contrast, we obtained evidence indicating that Cajal bodies are not essential for mTR trafficking to telomeres in mouse cell lines. Here, we see mTR at telomeres in cell lines that exhibit no mTR accumulation within Cajal bodies (Figure 4.4A, and see also Figures 4.2 and 4.3) and in cells in which coilin expression (and thus intact Cajal bodies) has been knocked out (Figure 4.5), suggesting that mouse cells utilize a different route to deliver telomerase to its functional site. Consistent with that idea, we see mTR foci linking together in close association to form chains throughout S phase, with at least one of the foci in these chains overlapping with a telomere (Figure 4.6). The timing of these events corresponds to the timing observed for mTR-telomere associations (compare graphs in Figure 4.4B and Figure 4.6B), suggesting the mTR chains may serve in some capacity in the trafficking of telomerase to the telomere. Conceivably, these mTR chains are similar to the Cajal body associated foci we observed for human cells (15). The appearance of these foci also coincided with telomere localization, and led to the idea that Cajal bodies were playing a role in transporting human TR to telomeres (14, 15).

The development of a FISH procedure specific for the detection of mTR opens the door to the wealth of genetic tools available for mice. By performing mTR FISH on cell lines derived from genetic nulls of genes implicated in telomerase function, we will be able to ascertain factors that specifically mediate the trafficking of telomerase to telomeres and hopefully gain a better understanding of how this process is regulated,

including how the cell cycle governs the trafficking pathway. Further, we can adapt and utilize this mTR FISH procedure to understand developmental changes in telomerase localization.

Materials and Methods

Cell culture

MEF-26 (WT), MEF-42 (coilin -/-), MEF-14 (mTR -/-) (a gift of Carol Greider, Johns Hopkins University, Baltimore, MD), 3T3 (ATCC, Manassas, VA), n2a (a gift of Brian Condie, University of Georgia, Athens, GA), c2c12 (ATCC), and A9 cells were grown on coverslips in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (FCS) (Mediatech) at 37˚C with 5% CO_2.

FISH probes

All probes were synthesized by Qiagen (Valencia, CA) as follows: mTR 117-169 (probe 1), CT*CCCGGCGAACCT*GGAGCTCCTGCGCT*GACGTTTGTTTTT*GAGGCTCGGGT*A; mTR 296-342 (probe 2), CT*CGGGGACCAGT*TCCATTCCTGT*CCTTGCGGCGCT*CGCCCGGCCT*G; mTR 224-282 (probe 3), GT*GCCCCGCGGCT*GACAGAGGCGAGCT*CTTCGCGGCGGCAGCGGAGT*CCTAAGACCCT*A; mTR 57-104 (probe 4), CT*CTGCAGGTCT*GGACTTTCCT*GGCCCGCTGGAAGT*CAGCGAGAAAT*A; U3 (probe 5), TT*CAGAGAAACTTCTCT*AGTAACACACTAT*AGAACTGATCCCT*GAAAGTATAGT*

C; mU85 (probe 6),

AT*TACCAAAGATCT*GTGTGTCATCT*CTCAGTGGCCAT*GACACAGCTAAGT*C;

telomere (probe 7),

CT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*A (T* indicates aminoallyl-modified thymidines). All mTR probes (probes 1-4 above) were conjugated with cy3 mono-functional reactive dye according to the manufacturers protocol (GE Healthcare, Little Chalfront, Buckinghamshire, United Kingdom). The remaining probes were labeled with Alexa Fluor 647 or Oregon green dye according to the manufacturers protocol (Molecular Probes/Invitrogen, Carlsbad, CA).

mTR FISH

mTR FISH was performed essentially as described (http://www.singerlab.org/protocols, [15]). Hybridizations were carried out overnight at 37˚C; 200 ng each of two of the cy3 labeled mTR probes above were used per coverslip (most often probes 1 and 2 above). 5-10 ng Oregon green or Alexa Fluor 647 U3 (probe 5), 100 ng Oregon green mU85 (probe 6), or 10 ng Oregon green telomere (probe 7) may have been included in the hybridization as well. Cells were then rinsed twice in 50% formamide, 2X SSC for 30 minutes at 37˚C. Cells were subsequently washed once in 50% formamide, 2X SSC, 0.1% NP-40 for 30 minutes at 37˚C. The coverslips were then mounted in Prolong gold mounting media (Molecular Probes/Invitrogen), cured at room temperature for 1 hour and stored at -20˚C until microscope analysis.

RNAse treatment

After fixation and permeabilization, cells were rehydrated in 1X PBS containing 1.5 mM $MgCl_2$ at room temperature for 5 min. Then the cells were incubated with RNAse A (0.2

mg/ml in 1X PBS containing 1.5mM $MgCl_2$) at 37°C for 2 h. mTR FISH was performed after the cells were washed 3 times with 1X PBS and once with 50% formamide, 2X SSC.

Indirect Immunofluorescence (IF)

Following FISH, cells were washed three times with 1X PBS. Next, cells were incubated with one of the following primary antibodies at the indicated dilution for 1 hour at room temperature: rabbit anti-Nopp140 (1:1,000, a gift from U. Thomas Meier, Albert Einstein College of Medicine, New York, NY), rabbit anti-TRF1 (1:500), rabbit anti-TPP1 (1:500), or rabbit anti-Tin2 (1:500)(all 3 a gift from Susan Smith, Skirball Institute, New York, NY). Cells were washed three times in 1X PBS and then incubated with secondary antibody (1:100 Cy2 conjugated goat anti-rabbit IgG (H+L), Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. All antibodies were diluted in 0.05% Tween-20 in PBS (PBST). Cells were then subjected to three final 1X PBS washes and mounted as above.

For the following antibodies IF was performed prior to FISH analysis: mouse anti-p80 coilin (1:1,000), mouse anti-Fibrillarin (17C12, 1:1,000), mouse anti-SMN (2B1, 1:100, Novus Biologicals, Littleton, CO). Briefly, cells were grown on coverslips overnight and then washed one time in 1X PBS. Cells were fixed in 4% formaldehyde in 1X PBS for 10 minutes at room temperature. Cells were rinsed twice in 1X PBS and blocked in 1% BSA (Sigma-Aldrich, St. Louis, MO) in 1X PBS for 1 hour at room temperature. Cells were then incubated with the above antibodies at the indicated dilutions for 1 hour at room temperature followed by incubation with a cy2 conjugated goat anti-mouse secondary antibody (1:100, Jackson Immunoresearch Laboratories) for 1 hour at room

temperature. These antibodies were diluted in 1% BSA in 1X PBS. Following 3 1X PBS washes, cells were again fixed in 4% formaldehyde in 1X PBS for 10 minutes at room temperature. Cells were rinsed twice in 1X PBS and once in 50% formamide, 2X SSC prior to FISH analysis.

S phase synchronization

Synchronous populations of MEF and 3T3 cells were obtained by double thymidine block. Cells were treated with 2 or 5 mM thymidine (Sigma-Aldrich) for 16 hours. Cells were released by rinsing twice with 1X PBS and replacing the normal growth media for 8 hours. Cells were retreated with 2 or 5 mM thymidine for another 16 hours. At various time points after release, cells were fixed and analyzed by PCNA staining and FACS analysis to verify cell cycle phase.

PCNA (proliferating cell nuclear antigen) staining

To distinguish S phase cells, cells on coverslips were rinsed once in 1X PBS and fixed for 10 minutes at room temperature in 4% formaldehyde, 10% acetic acid in 1X PBS. Cells were rinsed twice in 1X PBS and permeablized in 70% ethanol at 4˚C overnight. The cells were incubated with mouse anti-PCNA antibody (PC10, 1:2500, Abcam, Cambridge, MA) for 1 hour at room temperature. Cells were rinsed 3 times in 1X PBS and then incubated with secondary antibody (1:100, AMCA-conjugated goat anti-mouse IgG (H+L), Jackson Immunoresearch Laboratories) for 1 hour at room temperature. Cells were rinsed 3 times in 1X PBS and fixed in 4% formaldehyde in 1X PBS for 10 minutes at room temperature. Cells were rinsed twice in 1X PBS and once in 50% formamide, 2X SSC prior to FISH analysis. Both antibodies diluted in PBST.

FACS (fluorescence activated cell sorting) analysis

Cells were collected and resuspended to a single cell suspension in 0.5 mL 1X PBS. The cell suspension was transferred to a tube containing 4.5 mL of 70% ethanol and stored at -20˚C until analysis (at least overnight). On the day of FACS analysis, the ethanol suspended cells were spun down for 5 minutes at 200 x g. The pellet was washed once in 5 mL of 1X PBS, spun down again for 5 minutes at 200 x g and resuspended a solution of 0.02 mg/mL propidium iodide (Sigma-Aldrich), 0.1% Triton X-100, and 2 mg DNase-free RNAse A in 1X PBS. The cells were stained in this solution for 30 minutes at 37˚C. FACS analysis was performed using a FACSCALIBUR scanner (Becton Dickinson Biosciences, San Jose, CA) and data was analyzed using FlowJo software (Tree Star, Inc., Stanford, CA).

Microscopy

Slides were analyzed using a Zeiss Axioskop 2 Mot Plus fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were acquired at 63x (Plan Apochromat objective, numerical aperture 1.4) using a cooled charge-coupled device Orca-ER camera (Hamamatsu, Bridgewater, NJ) and IPLab Spectrum software.

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Figure 4.1. Mouse telomerase RNA is found in small spherical foci within the nuclei of cultured mouse cell lines. *A. Schematic structure of mTR.* The predicted secondary structure of mTR is shown (37). Black bars indicate the regions encompassed by each oligonucleotide probe. Asterisks denote the two probes (probes 1 and 2) used throughout this manuscript. *B. FISH procedure specifically detects the presence of mTR.* mTR FISH was performed on wild type MEF cells (WT, RNAse panels) or MEF cells derived from mTR -/- mice (mTR KO panels). Arrowheads denote intranuclear mTR foci present in the WT cells (WT panels), which are lost upon treatment of cells with RNAse A prior to FISH (RNAse panels). DAPI was used as a nuclear stain. Scale bar, 10 microns.

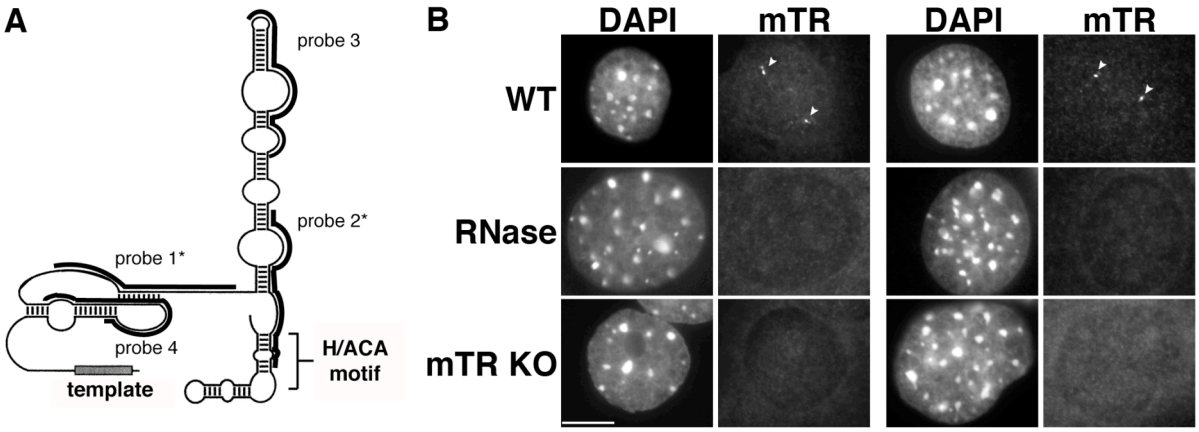

A
probe 3
probe 2*
probe 1*
probe 4
template
H/ACA
motif
B
DAPI
mTR
DAPI
mTR
WT
RNase
mTR KO

Figure 4.2. mTR foci do not colocalize with coilin in a variety of mouse cell lines. Mouse embryonic fibroblast (MEF, 3T3), n2a neuroblastoma, and c2c12 myoblast cell lines were co-analyzed for mTR (detected by FISH, red) and coilin (marker protein for Cajal bodies, detected by IF,green). Merge panels indicate an overlay of mTR and coilin panels. Open arrowheads point to Cajal bodies that do not overlap with mTR foci. Scale bar, 10 microns.

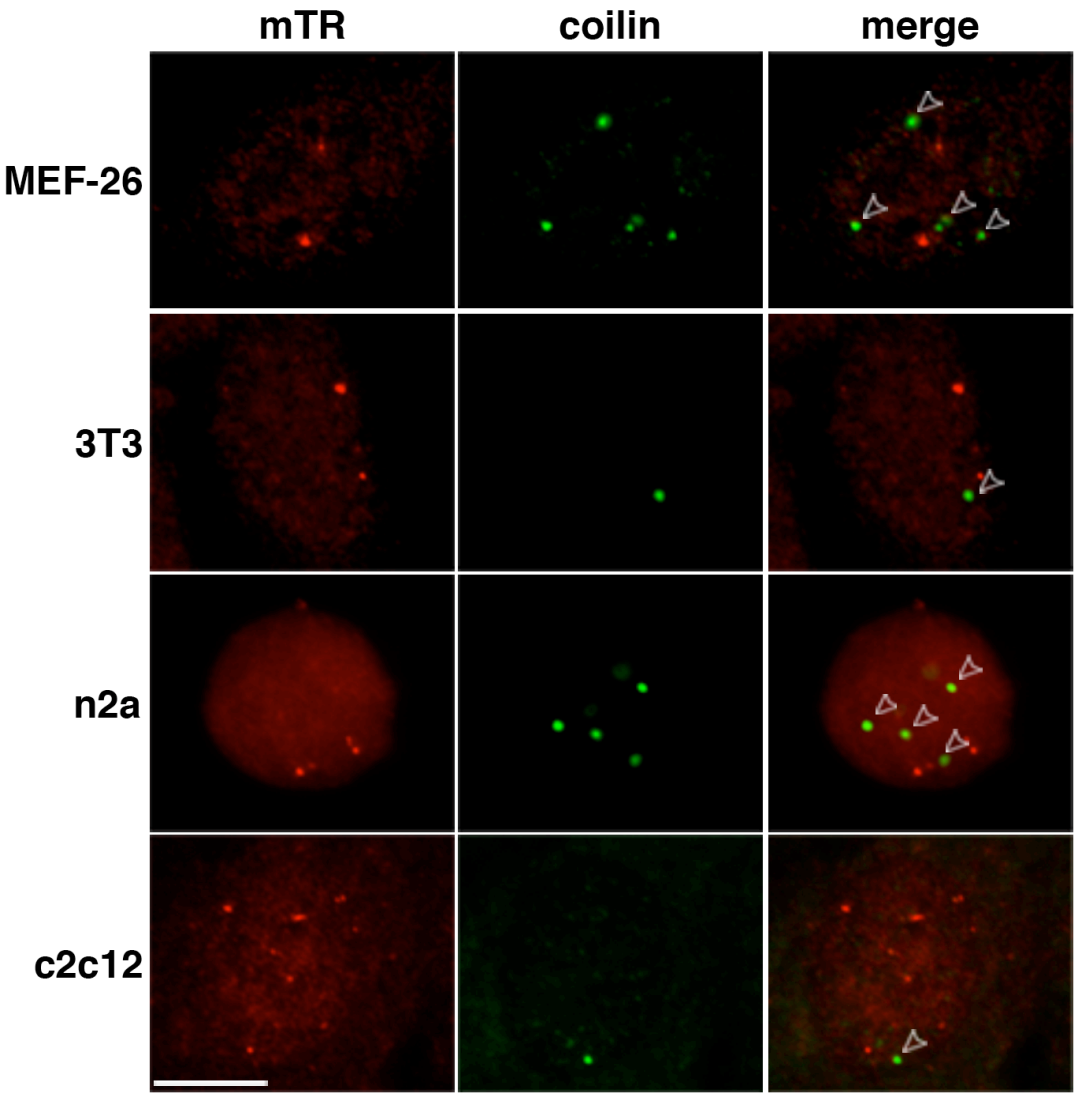

mTR
coilin
merge
MEF-26
3T3
n2a
c2c12

Figure 4.3. mTR resides in foci separate from Cajal bodies. *A. mTR foci do not correspond to known markers for Cajal bodies.* mTR FISH (red, mTR panels) was performed in tandem with one of three markers for Cajal bodies: U85 scaRNA (top row, detected by FISH), SMN (middle row, detected by IF, signal present in Cajal bodies and cytoplasm), or Nopp140 (bottom row, detected by IF, signal present in Cajal bodies and nucleoli). Arrowheads denote Cajal bodies; open arrowheads point to mTR foci that do not localize to Cajal bodies. *B. mTR localizes to intranuclear foci in MEF cells derived from coilin KO mice.* mTR FISH was performed on coilin KO MEF cells. Open arrowheads point to mTR foci. DAPI was used to stain the DNA. Scale bars, 10 microns.

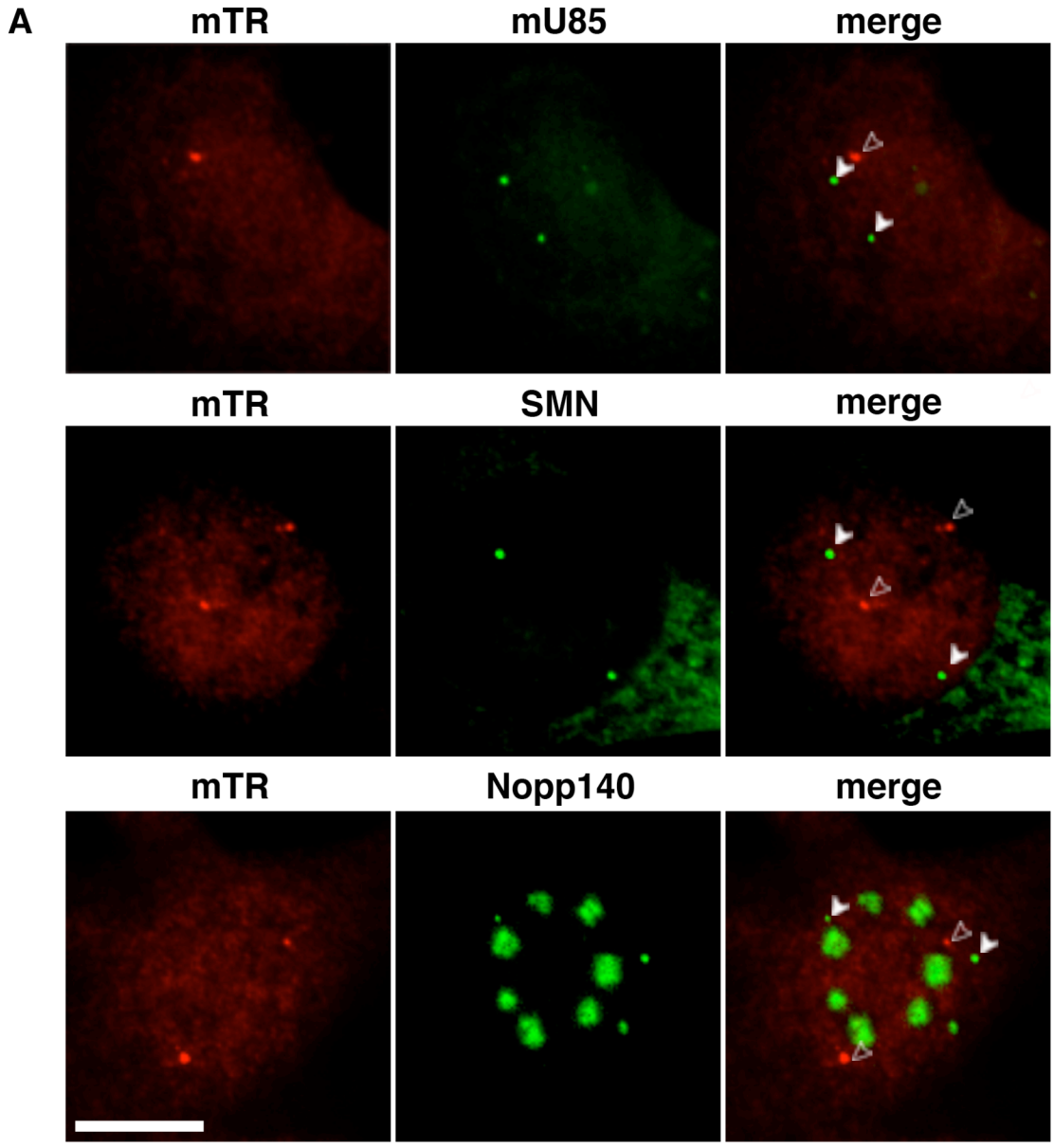

A
mTR
mU85
merge
mTR
SMN
merge
mTR
Nopp140
merge
B
DAPI
mTR
coilin KO

Figure 4.4. mTR localizes to subsets of telomeres in S phase. *A. Localization of mTR to telomeres occurs throughout S phase.* mTR FISH (red) and TIN2 IF (green, marker for telomeres) were performed on 3T3 cells at various stages of the cell cycle. PCNA staining was performed to identify S phase cells, as well as S sub-phase (i.e. early, mid, or, late, as indicated). Merge panels show a superimposition of mTR and TIN2 panels, yellow indicates an overlap of signal. Foci where both mTR and telomeres are present are indicated by arrowheads. Open arrowhead (in G1/G2 panels) denotes mTR focus that does not overlap with a telomere. Scale bar, 10 microns. *B. The frequency of mTR-telomere associations increases as cells progress through S phase.* The percentage of 3T3 cells with mTR foci that display mTR at the telomere is plotted relative to time after release from a double thymidine block. A, asynchronous cells. Data represents a compilation of two separate experiments.

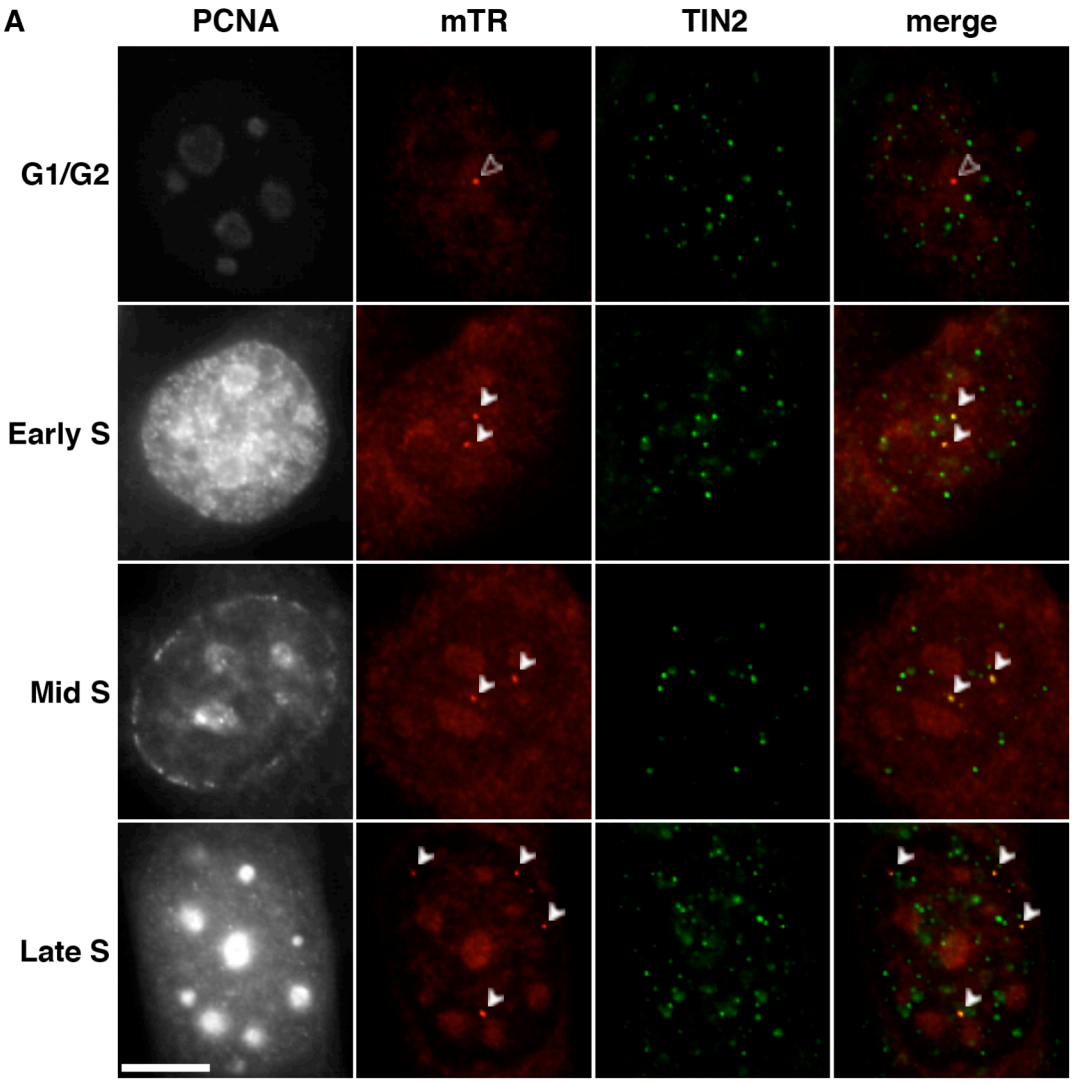

A
PCNA
mTR
TIN2
merge
G1/G2
Early S
Mid S
Late S
B
% cells with mTR foci that display mTR-telomere associations
40
35
30
25
20
15
10
5
A
0h
1h
2h
4h
6h

Figure 4.5. mTR localizes to the telomere in coilin KO MEFs. mTR FISH (red) and TPP1 IF (green, marker for telomeres) were performed on MEF cells derived from wild type (WT) and coilin -/- (coilin KO) MEFs. Merge panels display an overlay of mTR and TPP1 signals. Arrowheads denote foci where both mTR and TPP1 signals overlap. Scale bar, 10 microns.

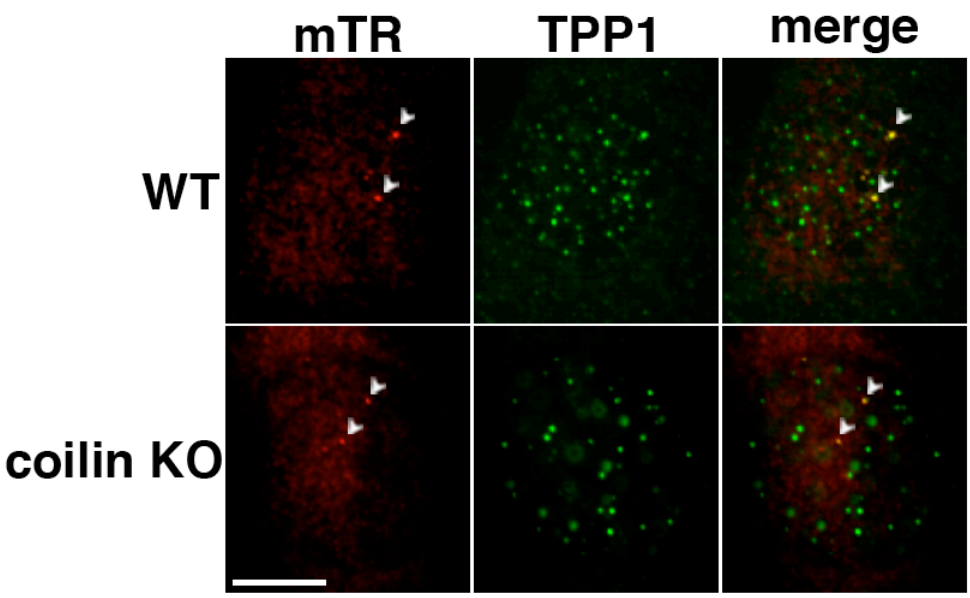
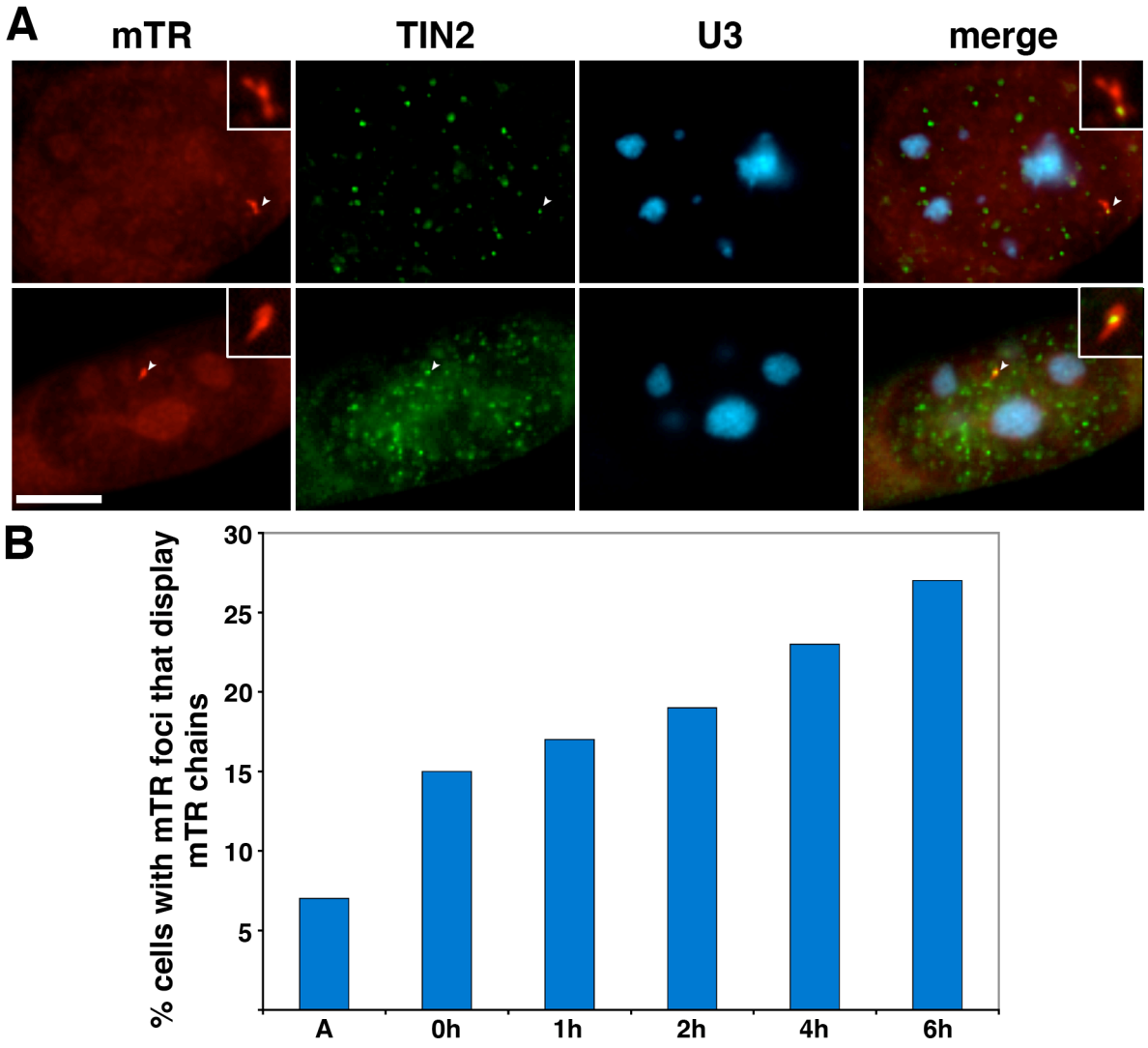

mTR
TPP1
merge
WT
coilin KO

Figure 4.6. mTR foci join together during S phase of the cell cycle. *A. mTR foci link together to form chains that are frequently associated with telomeres but not nucleoli.* 3T3 cells were stained for mTR (detected by FISH, red panels), TIN2 (detected by IF, green panels), and U3 snoRNA (detected by FISH, blue panels). Merge panels display a superimposition of all 3 panels. Insets show an enhanced magnification of the mTR chains. Arrowheads point to mTR foci within the chain that overlap with a telomere. Scale bar, 10 microns. *B. The frequency of mTR chains gradually increases over S phase of the cell cycle.* The percentage of 3T3 cells with mTR foci that display a mTR chain are plotted relative to time after release from a double thymidine block. A, asynchronous cells. Data compiled from two separate experiments.


A
mTR
TIN2
U3
merge
B
% cells with mTR foci that display mTR chains
30
25
20
15
10
5
A
0h
1h
2h
4h
6h

Chapter 5

Discussion

Control of telomere length is a highly regulated process in human cells. On the one hand, cells must have a sufficient reserve of telomeric DNA at their chromosome ends to prevent the onset of cellular senescence and premature aging (1). On the other, the progressive shortening of telomeres exhibited by human cells is thought to function in a tumor-suppressive manner, limiting the replicative capacity of cells (2). Maintaining a balance between these two extremes (lengthening and shortening) is critical for long-term cellular survival.

Regulation of telomerase (the RNP enzyme that synthesizes telomeric repeats) function is essential for achieving telomere length homeostasis. In humans, telomerase is expressed in all tissues early in development and is subsequently downregulated in the majority of tissues (exceptions being highly proliferative tissues such as bone marrow stem cells), due to a termination of transcription from the hTERT promoter (3). In fact, transcriptional control of hTERT is the primary means of telomerase regulation employed by cells (4).

Other questions about telomerase regulation, namely how and where within the cell telomerase is assembled from its core components TR and TERT as well as how the enzyme travels from its site of biogenesis to its site of function, have been the focus of our studies (Chapters 2-4, Appendices 1 and 2 [5-7]). Our work (along with that of others [8, 9]) has shown that telomerase exhibits dynamic changes in localization and implicates a multitude of nuclear structures in the biogenesis and trafficking of telomerase.

Telomeres are synthesized (by telomerase) exclusively during S phase of the cell cycle, corresponding to the timing of DNA synthesis ([10, 11] but see also [12]). In

yeast, a specific protein, Est1p, enters the telomerase complex (consisting of TLC1 and Est2p [TR and TERT, respectively]), which is present at the telomere throughout interphase, during S phase to activate the enzyme (Figure 1.5A) (13). The data presented here provide new insight into how telomerase is restricted to function only during this window of the cell cycle in mammalian cells. In Chapters 2-4 we clearly show that components of telomerase are associated with subsets of telomeres specifically during S phase (Figures 2.2B, 2.3C, 2.4, 3.5, 3.6, 4.4), suggesting the regulation occurs at the level of subnuclear trafficking of the two core telomerase components. The components of telomerase are sequestered away from the substrate telomere throughout the majority of the cell cycle (Figures 2.1, 2.3A, 4.4), most likely to prevent telomerase from functioning at nontelomeric sites (i.e. double strand breaks). In line with this, ectopically expressed TERT has been shown to move from the nucleoplasm (site of DNA, and telomeres) into nucleoli in response to induction of DNA breaks by ionizing radiation (14).

Elegant studies in yeast have shown that not every telomere is extended within a given cell cycle (15). Here, we provide evidence that this phenomenon is very likely occurring in human and mouse cells as well, as telomerase is only found at a small fraction of telomeres within any given S phase cell (Figures 2.2B, 2.3C, 3.6, 4.4). Why only specific subsets of telomeres recruit telomerase is thought to be mediated by telomere length. While our studies do not directly address this idea, several lines of evidence suggest that we are viewing telomerase at the shortest telomeres in the population. In mice, when strains with long telomeres are crossed with those with short telomeres, the shorter telomeres are preferentially elongated (16). Also, when

telomerase levels are limiting (in both human and mouse), the shortest telomeres are preferentially elongated (thus the shortest telomere, not bulk telomere length, determines replicative capacity) (17, 18). The most conclusive evidence comes from yeast, where the analysis of the elongation of single telomeres showed telomerase preferentially acted on the shorter telomeres (15). Thus, shorter telomeres are predicted to adopt a state whereby they are more "extendible" than longer telomeres (which are in more of a "nonextendible" state) (15). The telomeres in this "extendible" state more than likely adopt a chromatin structure that is more conducive to telomerase recruitment (i.e. they are probably not forming a t-loop structure, see Figures 1.2 and 1.7).

In Chapter 3, we show that TERT expression is essential for targeting TR to telomeres during S phase (Figure 3.6 and see Figure 3.4). Also, TR enhances the association of TERT with telomeres (Figure 3.7 A and B). Together these data suggest that trafficking of telomerase to telomeres is tied to biogenesis of the enzyme. Moreover, we show that increasing the levels of both components leads to an increased number of telomerase-telomere associations per cell (Figures 3.6B and 3.7A). In line with this, overexpression of telomerase (both TR and TERT) leads to extensive elongation of telomeres in a manner that is no longer dependent on telomere length (19). Taken together, this suggests that limiting levels of telomerase allow the enzyme to be more sensitive to changes in telomere length, ensuring the shortest telomeres recruit telomerase and are elongated first (and thus will not reach a critically short length). How this sensing mechanism manifests itself within the cell is not clear. Presumably, all telomeres are in an open chromatin state at some point during S phase

to allow for DNA replication at the telomeric ends. These structural changes in the telomeric chromatin could conceivably allow telomerase to have access to the telomere as well. Perhaps longer telomeres more readily adopt the closed ("nonextendible") chromatin state following replication events and thus do not recruit telomerase as often as the shorter telomeres in the population (which remain in the open, "extendible" state longer).

It is not yet known whether the intranuclear movement of telomerase to telomeres results from passive diffusion of telomerase components or involves some sort of molecular motor. However, it is likely the trafficking of telomerase is mediated by several trans-acting factors. Presumably components of the shelterin complex (Figure 1.2B) modulate the telomeric chromatin so that the 3' end of the telomere is accessible for extension by telomerase. TPP1 is the only shelterin component shown to directly interact with telomerase (to date), and it is thought to regulate telomerase function at telomeres (although how is still not clear) (20, 21). Telomerase also binds to numerous other proteins that likely enter/exit the telomerase RNP in order to regulate its function and/or trafficking. Telomerase RNA is stably bound by the four core H/ACA snoRNP proteins through its H/ACA motif (22-25), and this motif is required for nuclear retention of the RNA (5). Binding of these four core proteins to hTR is essential for RNA stability (22-25), but it is not yet clear if the H/ACA proteins moderate the localization of telomerase to telomeres (or if they affect telomerase trafficking in general). In addition, human homologs to yeast Est1p (the protein that activates yeast telomerase during S phase), EST1A and EST1B, have been identified and also associate with telomerase (26, 27). How the human EST1 proteins function in modulating telomerase activity is

not yet understood. The use of our mTR FISH procedure (see Chapter 4) on mouse cell lines derived from genetic knockouts of these and various other telomerase-associated proteins will be critical towards determining how these and other factors (including cell cycle regulators) govern the trafficking of telomerase to telomeres.

Throughout the cell cycle, hTR is found within intranuclear structures called Cajal bodies in telomerase-positive cancer cell lines (Figures A1.3, A1.4, and 2.1 [7-9]), while no accumulation is observed in telomerase-negative normal (or ALT, telomerase-negative cancer) cells (Figures A1.2, 3.1, and 3.2 [7]). Localization of hTR to Cajal bodies is dependent on hTERT, as expression of the protein is both necessary (Figure 3.4) and sufficient (Figure 3.3) for the localization event. Ectopically expressed hTERT also localizes to Cajal bodies (Figure 3.8 and [7]), leading us to postulate that the Cajal body is the site of telomerase assembly. Using the *Xenopus* oocyte system, we were able to more directly ascertain when and where vertebrate telomerase biogenesis likely occurs (Appendix 2). RNAs injected into the nuclei of oocytes behave as newly transcribed RNAs, allowing us to view early steps in telomerase RNP assembly. Injected TR molecules rapidly assemble with endogenous TERT (Figure A2.4), correlating with the time the RNA is found within Cajal bodies (Figure A2.3).

Taken together, our data (from these two systems) suggest that telomerase is assembled within Cajal bodies. However, we do not detect TR within Cajal bodies in the majority of cultured mouse cell lines (Figures 4.2 and 4.3). Instead, mTR resides in separate nuclear foci throughout the majority of the cell cycle. The identity of these foci remains unknown, but they appear to be a telomerase-specific body (as they do not colocalize with snoRNAs or scaRNAs, etc., Figure 4.3).

Similarly, coilin and SMN (the survival of motor neurons protein), the primary markers for Cajal bodies and gems (short for gemini, or twins, of Cajal bodies), respectively, can also be found in separate foci in some cell types (28, 29). Typically, Cajal bodies and gems are coincident structures in the nucleus (similar to the telomerase foci and Cajal bodies); this interaction is mediated by methylation of the coilin protein (30). Localization of SMN (and gems) to Cajal bodies appears to be developmentally regulated. In fetal tissues, the two are found as separate structures, whereas SMN and coilin colocalize in adult tissues (31, 32). Likewise, the localization of telomerase RNA to Cajal bodies may be under developmental control. The cell lines in Figure 4.2 are either fetal in origin or are in an undifferentiated state. Further testing needs to be done to discern why mouse TR is not within Cajal bodies (and to see if the process of Cajal body localization is developmentally regulated).

Besides serving as a site of telomerase assembly, Cajal bodies are likely involved in the S phase-specific trafficking of telomerase to telomeres. Coincident with the timing of hTR at telomeres, we see the emergence of TR- and TERT-containing Cajal body-associated foci (Figures 2.4 and 2.5). These foci emerge as hTR is compartmentalized to one (or two) poles of the Cajal body (Figure 2.5). Some of these foci directly overlap with a telomere ([9] and our unpublished data). In addition, Cajal bodies (containing hTR) have been shown to move throughout the nucleus to telomeres during S phase (9). In line with this, hTR CAB box mutants, which transiently associate with but do not stably reside within Cajal bodies ([8] and see Figure A2.6C), are functionally deficient in telomere elongation *in vivo* (33). These mutants are capable of forming catalytically active telomerase (Figure A2.6A and [33, 34]), but show an

impaired ability to associate with telomeres (compared to wild-type telomerase RNA), indicating the stable retention of hTR within Cajal bodies is important for telomerase trafficking to telomeres and ultimately for telomere extension (33). Together, these data suggest Cajal bodies serve to deliver telomerase to the telomeres. This delivery likely requires ATP, as the intranuclear movement of Cajal bodies is an energy-dependent process (35).

However, Cajal bodies appear not to be strictly required for transit of telomerase RNA to telomeres, and may instead serve to enhance the interaction. TR is capable of reaching the telomere in cells that are normally devoid of well-formed Cajal bodies (Figure 3.6). We also see mTR at telomeres in cell lines that display no mTR within Cajal bodies (Figures 4.2., 4.3, and 4.4). Further, coilin (the hallmark protein of Cajal bodies) is not required for the interaction (Figure 4.5). Instead, a different mechanism appears to have evolved to transport TR to the telomere in mouse cells, one that involves a linking together of multiple telomerase foci (Figure 4.6). These chains of mTR may be similar to the compartmentalization of TR and TERT into foci adjacent to the Cajal body in human cells (Figure 2.5).

These Cajal body-adjacent foci may also represent an S phase-specific site for telomerase biogenesis. In contrast to what we see for hTR, we do not detect significant levels of endogenous TERT protein in Cajal bodies throughout the cell cycle (Figures 2.3 and 2.4). These adjacent foci represent the first common place we see endogenous TR and TERT during their S phase trafficking (Figures 2.4, 2.5, and 2.6).

We also see a fraction of TR and TERT localizing to separate compartments of the nucleolus during early S phase of the cell cycle (Figures 2.2A and 2.3B).

Intriguingly, we see these components at only one nucleolus in a given cell. The significance of this localization pattern is not known, but the nucleolus is likely not the site of telomerase assembly. In Appendix 2, we clearly show that telomerase can assemble independent of nucleolar localization. Assembly of TR and TERT occurs prior to localization of the RNA within nucleoli (Figures A2.3 and A2.4). Also, mutation of the H/ACA motif of TR (which is essential to localize the RNA to nucleoli [5]) does not prevent enzyme assembly (Figure A2.5). Nucleoli are enriched in components that regulate the cell cycle and cellular proliferation, a number of which could function to regulate telomerase activity within the cell. Further research needs to be done to more precisely discern what role the nucleolus is playing in the biogenesis and trafficking of telomerase.

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Appendix 1

Telomerase RNA Accumulates in Cajal Bodies in Human Cancer Cells[1]

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[1]Yusheng Zhu, Rebecca L. Tomlinson, Andrew A. Lukowiak, Rebecca M. Terns, and Michael P. Terns. 2004. *Molecular Biology of the Cell*, Vol. 15, Issue 1, 81-90. Reprinted with permission of publisher.

Abstract

Telomerase synthesizes telomeric DNA repeats at the ends of eukaryotic chromosomes. The RNA component of the enzyme (hTR) provides the template for telomere synthesis, which is catalyzed by telomerase reverse transcriptase (hTERT). Little is known regarding the subcellular localization of hTR and hTERT and the pathway by which telomerase is assembled. Here we report the first glimpse of the detailed subcellular localization of endogenous hTR in human cells, which we obtained by fluorescence *in situ* hybridization (FISH). Our studies have revealed a distinctive hTR localization pattern in cancer cells. We have found that hTR accumulates within intranuclear foci called Cajal bodies in all typical tumor-derived cell lines examined (in which telomerase is active), but not in primary or ALT cells (where little or no hTERT is present). Accumulation of hTR in the Cajal bodies of primary cells is induced when hTERT is ectopically expressed. Moreover, we report that hTERT is also found in Cajal bodies. Our data suggest that Cajal bodies are involved in the assembly and/or function of human telomerase.

Introduction

Telomeres are nucleoprotein structures that cap the ends of linear eukaryotic chromosomes. In all vertebrates, telomeres consist of tandem TTAGGG DNA repeats and associated proteins (1-3). The function of telomeres is to protect chromosomes from degradation, recombination, and end-to-end fusion (4-6). Maintenance of telomere length is critical for genomic stability. Telomeres are synthesized by telomerase, a unique DNA polymerase that minimally consists of telomerase RNA and reverse

transcriptase subunits (hTR and hTERT respectively in humans; [7-10]). Telomerase uses a short sequence within its integral RNA as a template for the synthesis of telomeric DNA by the hTERT subunit.

In humans, activation of telomerase appears to be an essential, limiting step in cellular immortalization and tumor progression. Although telomerase activity is not detected in most adult somatic tissues, telomerase activity is present in greater than 90% of >2000 malignant tumors examined (11). hTERT is normally the limiting determinant of telomerase activity. hTR is present in both normal and cancer cells (7, 12, 13). In contrast, hTERT is undetectable in most normal adult cells and tissues, but is readily detected in most cancer cells and primary tumors (8, 10, 14). Recently, trace levels of hTERT and telomerase activity have been detected in cultured human fibroblasts; however, the amount of telomerase present is insufficient to prevent steady erosion of telomere length during cell division (15). Most cancer cells maintain telomeres, and the ability to divide indefinitely, by upregulating hTERT expression and telomerase activity (11, 16-19). Moreover, ectopic expression of hTERT in a number of primary cells reinstates telomerase activity and immortalizes the cells (20-24).

Despite the importance of telomerase to chromosome maintenance, cellular immortalization, and oncogenesis, little is known about the essential steps in the generation of the enzyme from individual RNA and protein components including how or where within the cell hTR and hTERT assemble to form telomerase. The intracellular localization of YFP- or GFP-hTERT fusion proteins in human cell lines has recently been reported. These studies indicate that hTERT is found throughout the nucleoplasm and is concentrated in nucleoli (25-27). Interestingly, exogenous hTERT undergoes

changes in distribution between nucleoli and the nucleoplasm in response to cell cycle phase, expression of viral oncogenes (cellular transformation), and double-stranded DNA breaks (26).

The existing information about the trafficking and subcellular localization of vertebrate telomerase RNA within the nucleus is primarily from microinjection studies in Xenopus oocytes that addressed the early trafficking patterns of telomerase RNA. This work indicates that telomerase RNA localizes to Cajal bodies and nucleoli at various times (28, 29). The nucleolus is well known as the site of assembly of ribosomes (30). Cajal bodies are dynamic, spherical structures present in plant and animal cell nuclei (31-34) that have recently been implicated as sites of posttranscriptional RNA modification (35-39) and assembly of various RNPs (40). The microinjection studies revealed that the box H/ACA motif found in hTR and all other vertebrate telomerase RNAs (41, 42) plays a role in the subcellular localization of the RNA. The H/ACA motif is characteristic of the H/ACA RNAs, small nucleolar (sno)RNAs and small Cajal body (sca)RNAs that guide nucleotide modification (pseudouridylation) of pre-rRNA within the nucleolus, or of snRNAs in the Cajal body, respectively (43, 44). Like snoRNAs, telomerase RNA is not exported to the cytoplasm but is retained within the nucleus (28). The H/ACA domain of hTR has been shown to be responsible for retention of the RNA within the nucleus (the cellular compartment where telomere synthesis occurs) and localization to the nucleolus (28). In addition to its roles in localization, the H/ACA domain mediates the binding of four H/ACA snoRNA-binding proteins to hTR (dyskerin, GAR1, NHP2, and NOP10; [45-48]) and is important for the metabolic stability of the RNA (28, 42, 49).

The detailed subcellular localization of endogenous hTR in human cells has not been reported. The presence of hTR in a variety of cancerous, precancerous, and normal cells has been demonstrated by low-resolution *in situ* hybridization primarily of histological tissue sections (reviewed in [50]). These studies indicate that hTR is primarily or exclusively located in the nucleus. Subcellular fractionation of HeLa cells has also shown that the majority of hTR is in the nucleus and that a small percentage (~7%) is found in a biochemical fraction enriched in nucleoli (42).

In this work, we have developed a specific and sensitive fluorescence *in situ* hybridization (FISH) assay and have performed the first detailed analysis of the subcellular localization of hTR in normal and cancer human cell lines. We have found that hTR accumulates in Cajal bodies in telomerase-positive cancer cell lines but not in primary cell lines, which lack hTERT and telomerase activity, or in U2OS cells, an atypical cancer line that expresses hTR but not hTERT and maintains telomeres by an alternative mechanism (i.e., an ALT [alternative lengthening of telomeres] cell line; [51]). The accumulation and detection of hTR in nuclear foci including Cajal bodies appears to be dependent on hTERT expression and was detected in hTERT-transfected fibroblasts but not untransfected fibroblasts. We did not detect accumulation of hTR at PML bodies, gems, or telomeres. Finally, we have found that YFP-tagged hTERT also specifically localizes to Cajal bodies. These findings implicate the Cajal body as a site of telomerase biogenesis and/or function.

Materials and Methods

Cell Lines

Table A1.1 shows the cell lines and media used in these experiments. All cells were cultured at 37°C under 5% CO2.

hTR FISH

All probes were aminoallyl-T-modified deoxyoligonucleotides synthesized by OPERON (Operon/Qiagen, Valencia, CA). The T* in the probe sequences indicates aminoallyl-thymine. Antisense hTR probe 1 (5'-T*GCGCGCGGGGAGCAAAAGCACGGCGCCT*ACGCCCTTCTCAGTT*AGGGTTAGACA-3') is complementary to hTR nt 43–96 with an additional T at the 5' end. Antisense hTR probe 2 (5'-GCT*GACATTTTT*TGTTTGCTCT*AGAATGAACGGT*GGAAGGCGGCAGGCCGAGGCT*T-3') is complementary to hTR nt 128–183. hTR sense probe (5'-GAGT*CAATCCCAAT*CTGTTTTTT*ACCGGTGGTGGGGAGGGT*CCGGGTGGGAGGCGT*TG-3')is the same as nt 3–60 of hTR. The aminoallyl-modified dTs in the probes were chemically conjugated with Cy3 fluorophore (Cy3 monofunctional reactive dye; Amersham Pharmacia, Piscataway, NJ). FISH was performed essentially as described by Robert Singer (http://www.singerlab.org/protocols). Briefly, cells were grown on coverslips for 18–24 h, rinsed once with 1x PBS, and fixed with 4% formaldehyde (37% liquid stock from Electron Microscope Sciences, Fort Washington, PA), 10% acetic acid, 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4) for 10 min at room temperature. After two washes with PBS, cells were permeablized by treatment with 70% ethanol overnight at 4°C. Cells were rehydrated for 5 min at room

temperature in 2x SSC and 50% formamide and prehybridized with a solution of 10% dextran sulfate (Sigma, St. Louis, MO), 2 mM vanadyl ribonucleotide complex (GIBCO BRL, Gaithersburg, MD), 0.02% RNAse-free BSA (Sigma), 40 µg Escherichia coli tRNA (Roche, Nutley, NJ), 2x SSC, and 50% formamide for 1 h at 37°C. Cells were then hybridized for 4 h at 37°C in 40 µl of the prehybridization solution described above containing a total of 80 ng of fluorescently labeled DNA probe. After hybridization, cells were washed twice with 2x SSC, and 50% formamide for 30 min at 37°C. Slides were mounted in 90% glycerol, 1x PBS, 1 mg/ml p-phenylediamine, and 0.1 µg/ml DAPI.

RNAse Treatment

After permeabilization in 70% ethanol overnight, cells were rehydrated in 1x PBS containing 1.5 mM MgCl2 at room temperature for 5 min. The cells were incubated with RNAse A (0.2 mg/ml) at 37°C for 2 h. hTR FISH was performed after the cells were washed two times with 2x SSC, 50% formamide for 10 min at 37°C.

Indirect Immunofluorescence

Following hTR FISH, cells were washed again with PBS at room temperature twice for 10 min per wash. Cells were incubated with one or two of the following antibodies at the indicated dilution for 1 h at room temperature: R288, rabbit anti-p80 coilin (1:1000; [52]) or mouse anti-p80 coilin (1:100; [53]); 5E10, mouse anti-PML (1:10; [54]); mouse anti-SMN (1:1000; BD); rabbit anti-TRF2 (1:100; [55]); rabbit anti-TRF1 (1:100; [56]). Cells were washed with PBS three times for 10 min at room temperature followed by incubation with 1:100 Cy2-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmnoResearch, West Grove, PA), 1:50 Alexa Fluor 350–conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR), or 1:100 Texas Red–conjugated goat anti-mouse

IgG (Jackson ImmnoResearch) for 1 h at room temperature. All antibodies were diluted in PBST (0.05% Tween 20 in PBS). After three 10-min washes in PBS, slides were mounted as described above.

Transfection and Visualization of YFP-hTERT

HeLa cells were grown on coverslips for 18 h and then transiently transfected with 1 μg of either plasmid pYFP-hTERT (25) or control plasmid pEGFP-N1 (Clontech, Palo Alto, CA) using LipofectAMINE 2000 (Invitrogen) reagent according to the manufacturer's instruction. Twenty-four hours after transfection, cells were extracted in 0.5% Triton X-100 in CSK (10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM NaCl, 1 mM EGTA, 3 mM MgCl2) for 1 min at 4°C. Coverslips were washed three times in 1x PBS. Cells were then fixed in 4% formaldehyde in PBS for 10 min at room temperature and washed twice with 1x PBS, and indirect immunofluorescence with anti-p80 coilin antibodies was performed as described above.

Microscopy

Analysis was performed on a Zeiss Axiovert S100 inverted fluorescence microscope (Thornwood, NY). All images were acquired at 63x magnification using a cooled charge-coupled device camera (Quantix-Photometrics, Roper Scientific, Duluth, GA) and IP Lab Spectrum software (Scanalytics, Fairfax, VA).

Results

Detection of the Subcellular Localization of Human Telomerase RNA

We have established a FISH technique to determine the subcellular localization of hTR in human cells. We designed two probes complementary to different regions in

the 5' domain of hTR (Figure A1.1A and see MATERIALS AND METHODS). On hybridization with either probe, 1–5 bright foci were observed in the nuclei of HeLa cells. Maximal hTR signal was observed when both probes were used in combination (Figure A1.1B). A series of controls indicate further that the FISH signals observed in our experiments are specific to telomerase RNA. No foci were apparent in HeLa cells upon hybridization with a sense probe or after treatment of HeLa cells with RNAse A, showing that telomerase RNA, rather than the telomerase RNA gene, is being detected (Figure A1.1C). Furthermore, signals were not observed in VA13 cells, an unusual ALT cell line that lacks hTR transcripts (as well as hTERT; (57, 58); Figure A1.1C).

In the course of these studies, we found that hTR probes spanning nucleotides 37–44 gave false-positive FISH signals (i.e., occurring in hTR-negative VA13 cells). As previously noted (28), nts 37–44 of hTR (AUUUUUUUG) match a consensus Sm protein binding site found in ubiquitously expressed and relatively abundant spliceosomal snRNAs. The false-positive signals observed with probes that included this region colocalized with Sm proteins, indicating that those probes recognized snRNAs. These results suggest caution with the use of probes against nts 37–44 of hTR and reevaluation of previous *in situ* experiments employing full-length hTR probes (reviewed in [50]).

hTR Accumulates in Intranuclear Foci in Telomerase-positive Cancer Cells, But Not Telomerase-negative Primary Cells

Telomerase activity is readily detected in the majority of human cancer cells, but not in the majority of normal cells. Although hTERT is selectively expressed in most cancer cells, hTR is expressed in both normal and cancer cells (7, 12, 13). With this in

mind, we investigated whether the localization of hTR differed between cancer and normal cell lines.

First, to determine the generality of the pattern of hTR localization observed in HeLa (cervical adenocarcinoma) cells (Figure A1.1), we performed hTR FISH on MCF7 (breast carcinoma), H1299 (nonsmall cell lung carcinoma), A549 (lung carcinoma), PC-3 (prostate adenocarcinoma), JEG-3 (choriocarcinoma), and DU145 (prostate carcinoma) cancer cells (Figure A1.2A and unpublished data). As with HeLa cells, 1–5 hTR-containing foci were present in the nuclei of all the cancer cells examined.

To test whether similar hTR foci are also found in normal cell lines, hTR FISH was performed on primary somatic cell lines. Interestingly, no accumulation of hTR at nuclear foci was observed in the normal primary fibroblast cell lines BJ and IMR-90 or the smooth muscle cell line 2yo (Figure A1.2B). hTR levels are generally lower in normal cells than cancer cells (13, 59); however, the difference in localization pattern is not likely due to a simple difference in hTR levels. The amount of hTR present in particular primary cells that we examined (e.g., BJ and IMR90 fibroblasts) is statistically equivalent to the amount measured for DU145 cancer cells (59), where localization to foci is observed (unpublished data and see Figure A1.4). Thus, the total amount of hTR present in normal cells is not below the level that can be detected by our FISH procedure when localized as in cancer cells. The above data indicate that the distribution of hTR differs in normal and cancer cells; hTR accumulates within intranuclear foci in cancer cells (where telomerase assembly is presumably ongoing) but not in normal cell lines (where little or no telomerase is made).

hTR Accumulates in Cajal Bodies in Cancer Cells

We next sought to determine the identity of the spherical nuclear foci that accumulated hTR in the tumor-derived cells. These foci are similar in size and shape to several nuclear bodies, including PML (promyelocytic leukemia) bodies and Cajal bodies. We performed double-labeling experiments to simultaneously evaluate the localization of hTR (via FISH) and these nuclear bodies (via immunofluorescence using antibodies against specific marker proteins).

The PML (promyelocytic leukemia) protein is present in PML bodies and has been implicated in the processes of cell proliferation, apoptosis, and senescence (reviewed in [60-63]). Furthermore, specialized PML bodies, APBs (ALT-associated PML bodies), are found in telomerase-negative ALT cells (51, 64). However, as can be observed in Figure A1.3 (top panel), no colocalization of hTR and the PML protein was observed in HeLa cells, indicating that hTR does not accumulate in PML bodies.

Previously, we had shown that telomerase RNA associates with Cajal bodies in Xenopus oocytes (28). Cajal bodies are thought to serve as centers of biogenesis for several nuclear RNPs (reviewed in [31, 32, 34, 40]). It is currently hypothesized that the SMN complex (SMN and six other proteins of the gemin family) mediates the assembly of RNPs including spliceosomes, snoRNPs, and transcriptosomes within Cajal bodies (40, 65-67). SMN is the protein implicated in the fatal neuromuscular disease, spinal muscular atrophy (SMA; [68]). Recent reports indicate a physical association of telomerase and SMN (69, 70). In HeLa cells, there was a precise colocalization between hTR and the Cajal body marker protein, coilin (Figure A1.3, second panel), indicating that the hTR-containing foci are Cajal bodies.

We also investigated whether hTR is present within gems in related cell lines where these structures are observed. Gems are nuclear bodies that also contain the SMN complex (65-67). The name gems (for gemini or twin) was assigned based on the initial observation that these nuclear bodies were found to be immediately adjacent to or coincident with Cajal bodies (65). Subsequent localization studies found that gems and Cajal bodies are merged, indistinguishable structures that contain coilin and SMN in most human cell lines (including those used in this study except where noted; [71-73]). We have examined hTR localization in two special HeLa cell lines, in which gems are distinct structures that are enriched in SMN and coilin methylation is reduced (74). Specifically, we performed triple labeling of hTR, coilin, and SMN in HeLa-PV and HeLa-KN cells. In all cases, hTR accumulated in Cajal bodies, but never in gems (Figure A1.3, third panel), suggesting that hTR does not simply colocalize with SMN and that coilin methylation and SMN and are not likely required for recruitment of hTR to Cajal bodies.

We also tested whether any of the hTR-containing foci were associated with telomeres, the site where telomerase functions. Using antibodies against telomere-binding proteins TRF1 and TRF2, we found that hTR signals do not typically overlap with telomeres (Figure A1.3, lower panel and unpublished data). In a small fraction of cells, at a few telomeres, we did observe colocalization of hTR (within Cajal bodies; unpublished data). However, the low frequency of the colocalization made it difficult to distinguish from random colocalization of Cajal bodies with the numerous telomeres. We cannot exclude the possibility that low levels of hTR are present at telomeres (or

other sites) due to detection limits of the FISH approach. Our data suggest that hTR is present in Cajal bodies and that the Cajal bodies are not usually found at telomeres.

We then tested for localization of hTR to Cajal bodies (by hTR FISH and coilin immunofluorescence) in other telomerase-positive cancer cell lines derived from various tissue sources (Figure A1.4 and unpublished data). In all cancer cells investigated, each of the hTR foci corresponded to a Cajal body, consistent with what was observed in HeLa cells. Thus, accumulation of hTR in Cajal bodies appears to be hallmark of a variety of cancer cell types.

hTERT Expression in Normal Cells Induces Accumulation of hTR in Foci Including Cajal Bodies

Cancer cells express readily detectable levels of hTERT (and telomerase activity), but primary cells do not. To assess whether the difference in distribution of hTR in normal and cancer cells could be attributed to hTERT expression, we compared hTR localization in primary BJ fibroblasts and BJ cells that stably express ectopic hTERT protein (Figure A1.5A). hTR was found in nuclear foci in BJ-hTERT cells. At the same time, no foci were observed in primary BJ cells, indicating that hTERT expression can induce the accumulation of hTR in foci within the nucleus. Similar results were obtained with 2yo smooth muscle cells and hTERT-expressing 2yo cells (unpublished data). The expression of hTERT in primary cells likely increases the level of hTR (perhaps about twofold; [59]), and thus the appearance of hTR in foci could reflect increases in hTR levels as well as redistribution of the RNA. The expression of hTERT in these cells also initiates the assembly of telomerase, which may account for the redistribution of hTR.

Unlike cancer cells, primary human cells do not frequently display prominent Cajal bodies (75, 76) and hTR-containing foci observed in the BJ-hTERT and 2yo-hTERT cells generally did not colocalize with coilin, SMN, or PML proteins (unpublished data). Thus, in contrast to the foci observed in cancer cells, most of the hTR-containing foci in normal cells ectopically expressing hTERT did not correspond to Cajal bodies and currently remain unidentified. However, hTR colocalized with the infrequent Cajal bodies observed in BJ-hTERT and 2yo-hTERT cells, but not with those found in BJ or 2yo cells (Figure A1.5B and unpublished data). These results suggest that accumulation of hTR in Cajal bodies is dependent on hTERT expression. Finally, we also examined hTR localization in cancer cells that contain numerous Cajal bodies and express hTR but not hTERT. U2OS is an osteosarcoma-derived ALT line (77). We were unable to detect hTR in foci within the nuclei of these cells, despite the presence of numerous Cajal bodies (Figure A1.5C). Taken together, the results indicate the importance of hTERT expression on hTR accumulation in Cajal bodies.

hTERT Also Localizes to Cajal Bodies

Given that hTR is targeted to Cajal bodies in cancer cells where telomerase is active and hTERT is expressed, we asked if hTERT also localizes to these structures. Previous studies have shown that hTERT is present in nucleoli and is also distributed throughout the nucleoplasm (25-27). The high levels of YFP-TERT protein present in the nucleoplasm of transfected cells in our earlier analysis (25) obscured our ability to determine if hTERT was present in Cajal bodies. The use of mild preextraction conditions, which remove the soluble pool of nucleoplasmic YFP-TERT, revealed a clear association of hTERT with Cajal bodies in HeLa cells (in addition to nucleoli as

previously reported; Figure A1.6). Transfection of GFP alone results in no accumulation in any nuclear structure after preextraction (unpublished data), demonstrating that hTERT is also present in Cajal bodies.

Discussion

Formation of the telomerase enzyme depends on the intracellular interaction of hTR and hTERT. To understand the biogenesis and function of telomerase, knowledge of the subcellular localization and trafficking of these key telomerase components is required. hTERT is expressed in most cancer cells, but little or no hTERT is present in most somatic cells. In contrast, hTR is expressed in both primary and cancer cells. Here we describe a FISH procedure that, for the first time, has enabled determination of the detailed subcellular localization of endogenous telomerase RNA in human cells. A major finding is that hTR localizes to intranuclear foci called Cajal bodies in telomerase-positive cancer cells, but not in normal cells. The difference in hTR localization may reflect active production of telomerase in cancer cells.

Our results establish the Cajal body as a site on the pathway traveled by telomerase in human cells—a potential site of telomerase biogenesis or function. Here we consider the significance of the localization of telomerase to Cajal bodies in the context of our current knowledge.

Cajal Bodies as Sites of Telomerase Biogenesis

We consider it likely that the Cajal body is the site where hTR and hTERT interact to form an active enzyme. Our results show that both of the key components of telomerase, hTR and hTERT, are found in Cajal bodies (Figures A1.3 and A1.6).

Moreover, we find that hTR accumulates in Cajal bodies, specifically in cells where telomerase assembly is presumably ongoing (Figures A1.1, A1.2, A1.3, A1.4), consistent with the notion that the Cajal body is the site of assembly. No accumulation was observed in either tumor-derived (ALT) or normal cells in which hTERT is reduced or absent and telomerase is not readily detectable (Figures A1.2 and A1.5). Furthermore, ectopic expression of hTERT (and telomerase activity) in primary fibroblasts or smooth muscle cells resulted in accumulation of hTR in Cajal bodies (Figure A1.5). The hypothesis that telomerase assembly takes place within Cajal bodies is also based on considerable evidence implicating the Cajal body as the site of assembly of a variety of cellular RNPs (31-34, 40). An implication of our findings is that Cajal body localization of hTR could serve as a marker for telomerase-positive cells.

Within Cajal bodies, the assembly of hTR and hTERT into active RNP complexes may be mediated the SMN (survival of motor neuron) complex. This macromolecular complex consists of the SMN protein (the spinal muscular atrophy disease protein) and six additional protein components called gemins 2–7 and is present in Cajal bodies of most cells (65, 71, 72, 78). Growing evidence indicates that SMN chaperones the assembly of several cellular RNPs, including both snRNPs and snoRNPs (reviewed in [40, 66, 67]). Recent data suggest SMN may also play a role in telomerase biogenesis, because SMN directly interacts with the telomerase-associated protein GAR1 (70), and antibodies against SMN immunoprecipitate catalytically active telomerase (69).

Additional steps in telomerase biogenesis may also occur in the Cajal body. For example, hTR is associated with the H/ACA snoRNA-binding proteins dyskerin (47), GAR1 (46), NHP2, and NOP10 (45, 48) *in vivo*. The H/ACA snoRNP proteins are found

in Cajal bodies (32, 44, 45, 48) and may assemble with telomerase here. Recent studies also suggest that the Cajal body is the site of posttranscriptional modification (ribose methylation and pseudouridylation) of some RNAs by the guide RNAs that reside in this structure (scaRNAs, the small Cajal body RNAs; [35, 79]). Like most other stable cellular RNAs, hTR likely undergoes posttranscriptional nucleotide modifications that influence the stability and function of the RNA. Conceivably, hTR modifications occur within Cajal bodies.

Studies using the Xenopus oocyte system provided the first evidence that vertebrate telomerase RNA localizes to Cajal bodies. Fluorescently labeled human or Xenopus telomerase RNA is specifically targeted to Cajal bodies (and nucleoli) after microinjection of the RNA into Xenopus oocyte nuclei (28, 29). Taken together with the findings of this study (that hTR localizes to Cajal bodies in a variety of human cell lines), the evidence indicates that hTR localization to Cajal bodies is a common step in the biogenesis of vertebrate telomerase. Interestingly, ciliate telomerase RNA is also predominantly localized to spherical intranuclear foci, some of which may be Cajal bodies (80). The hTR-containing foci in ciliates contain hypermethylated RNA, a hallmark feature of Cajal bodies found in other eukaryotes (80). Thus, Cajal bodies appear to be a component of the telomerase biogenesis pathway for a variety of diverse eukaryotes.

Cajal Bodies and Telomerase Function

In telomerase-positive cells, active telomerase RNPs ultimately encounter telomeres to carry out telomere replication. In this study, we were generally unable to detect hTR at the telomeres. This may reflect the detection limit of the FISH procedure

given that only a few molecules of hTR may function at each telomere. In addition, hTR may only interact with these structures transiently. For example, hTR may localize to telomeres only during S phase when telomeres are synthesized (81, 82). Evidence for this notion comes from important work done in ciliates that showed that a small fraction of telomerase RNA is mobilized during S phase to the replication band, the site of telomere synthesis (80). At all stages of the cell cycle (including S phase) the majority of detectable ciliate telomerase RNA is present at intranuclear foci (that may include Cajal bodies; [80]).

In both cilates (80) and human cells (this study), much of the telomerase RNA appears to be in intranuclear structures and sequestered away from telomeres. Regulated movement of hTR (or assembled telomerase) out of Cajal bodies during S phase may be necessary for telomere synthesis. Several properties of Cajal bodies suggest that they may deliver telomerase to telomeres. Cajal bodies undergo cell cycle–related structural changes (75, 83-86), are highly motile structures (87-89), and are known to associate with specific chromosomal loci (reviewed in [32, 90]).

Telomerase and Nucleoli

Previous studies have proposed that the association of hTR and hTERT takes place within the nucleolus. A fraction of both hTERT (25-27) and hTR (28, 29, 42) localizes to nucleoli (as well as Cajal bodies). Furthermore, the nucleolus is well known as the site of assembly of the ribosome and perhaps other small RNPs (30, 91, 92). Although we did not detect hTR in nucleoli in this study, it is possible that hTR transiently localizes to nucleoli where assembly occurs.

A recent study indicates that the intranuclear distribution of ectopically expressed hTERT may shift between nucleoli and the nucleoplasm in response to a variety of stimuli (26). The authors propose that nucleolar localization provides a means to sequester active telomerase away from nucleoplasmic telomeres. The model is based on the assumption that the nucleolar hTERT is part of active telomerase complexes (and not unassembled hTERT molecules). However, nucleolar targeting of hTERT occurs in human cells that do not express hTR (25), indicating that unassembled hTERT accumulates in nucleoli. In addition, the fraction of hTR that may be in nucleoli is very small; it is undetectable by FISH (this study) and was estimated as ~7% of total hTR by biochemical fractionation analysis (42). Thus, it seems possible that the hTERT observed in nucleoli does not represent active telomerase.

It will be important to precisely elucidate the steps along the telomerase biogenesis pathway and to understand how each step is coordinated in the cell both temporally and spatially to control the availability of functional telomerase.

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Table A1.1. Human cell lines used in this study

Cell line	Description	Medium
HeLa	human cervix adenocarcinoma	Dulbecco's modified Eagle's medium (Sigma)
MCF7	human mammary gland adenocarcinoma	minimum essential medium Eagle's with 0.01mg/ml bovine insulin (ATCC)
H1299	human non-small cell lung cancer (ATCC No. CRL-5803)	RPMI 1640 (ATCC)
A549	human lung carcinoma	Ham's F12K medium (ATCC)
PC-3	human prostate adenocarcinoma	Ham's F12K medium (ATCC)
DU 145	human prostate carcinoma	minimum essential medium Eagle's (ATCC)
BJ	human normal foreskin fibroblast (ATCC No.CRL-2522)	minimum essential medium Eagle's (ATCC)
BJ-hTERT	BJ with hTERT stable transfection and SV-40 transformation	α-modification of Eagle's medium (Mediatech)
2yo	Human normal smooth muscle cell line	Endothelial cell basal medium-2 with supplements and growth factors (Clonetics)
2yo-hTERT	2yo with hTERT stable transfection (	Endothelial cell basal medium-2 with supplements and growth factors (Clonetics)
IMR-90	human normal lung fibroblast (ATCC No. CCL-186)	minimum essential medium Eagle's (ATCC)
VA13	SV40 transformed human lung fibroblast cell line WI38 subline 2RA (ATCC No.CCL-75.1)	Dulbecco's modified Eagle's medium (Sigma)
U-2 OS	human osteosarcoma	Dulbecco's modified Eagle's medium (Sigma)

Figure A1.1. Human telomerase RNA is present in intranuclear foci. (A) The predicted secondary structure of hTR is shown (adapted from [41]). Black bars denote regions of complementarity for each hTR probe. Probe 1 is complementary to hTR nts 43–96; probe 2 is complementary to nts 128–183. (B) Fluorescence *in situ* hybridization (FISH) of HeLa cells was performed with probe 1 (top), probe 2 (middle), or both (bottom). The combination of probes 1 and 2 is used in all subsequent figures except where noted. Fluorescence (hTR) and differential interference contrast (DIC) microscopy images are shown in this and subsequent figures. (C) Top panel shows FISH of HeLa cells performed with a sense probe. hTR FISH signal is lost upon treatment of HeLa cells with RNAse A before FISH (middle). There is no detectable accumulation of hTR in VA13 cells, which do not express hTR (bottom).

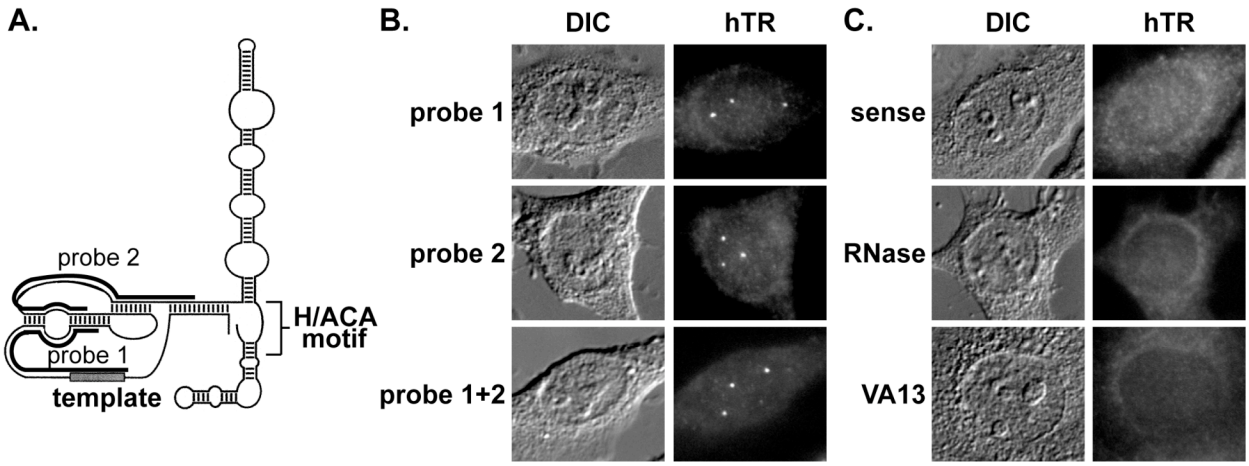

A.
probe 2
probe 1
template
H/ACA
motif
B.
DIC
hTR
probe 1
probe 2
probe 1+2
C.
DIC
hTR
sense
RNase
VA13

Figure A1.2. hTR accumulates in intranuclear foci in telomerase-positive cancer cells, but not telomerase-negative primary cells. (A) FISH analysis of hTR localization in the following telomerase-positive cancer cells is shown: MCF7 (breast carcinoma), H1299 (non-small cell lung carcinoma), A549 (lung carcinoma), and PC3 (prostate carcinoma). (B) FISH analysis of hTR localization in the following telomerase-negative primary cells is shown: BJ and IMR90 fibroblasts and 2yo smooth muscle cells.

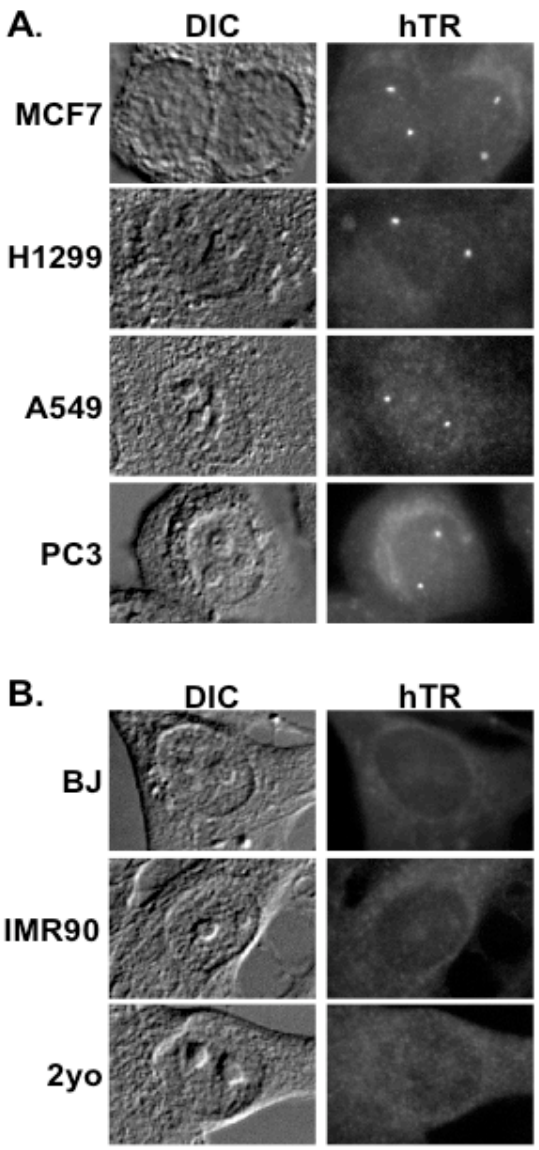

A.
DIC
hTR
MCF7
H1299
A549
PC3
B.
DIC
hTR
BJ
IMR90
2yo

Figure A1.3. hTR localizes to Cajal bodies. hTR FISH (red) was combined with indirect immunofluorescence (IF) using antibodies against the marker proteins for PML bodies (first row, PML, green) or Cajal bodies (second row, coilin, green) in HeLa cells. Merge panels show merged FISH and IF data; yellow indicates overlap of red and green signals. Merged data from an additional cell is shown in the last column. In the third row, hTR FISH (red) was combined with IF with both anticoilin (green) and anti-SMN antibodies (blue) to visualize Cajal bodies and gems in HeLa-PV cells. Second merge shows the analysis of HeLa-KN cells. In the fourth row, hTR FISH (red) was combined with IF with anti-TRF2 antibodies (green) to visualize telomeres in HeLa cells.

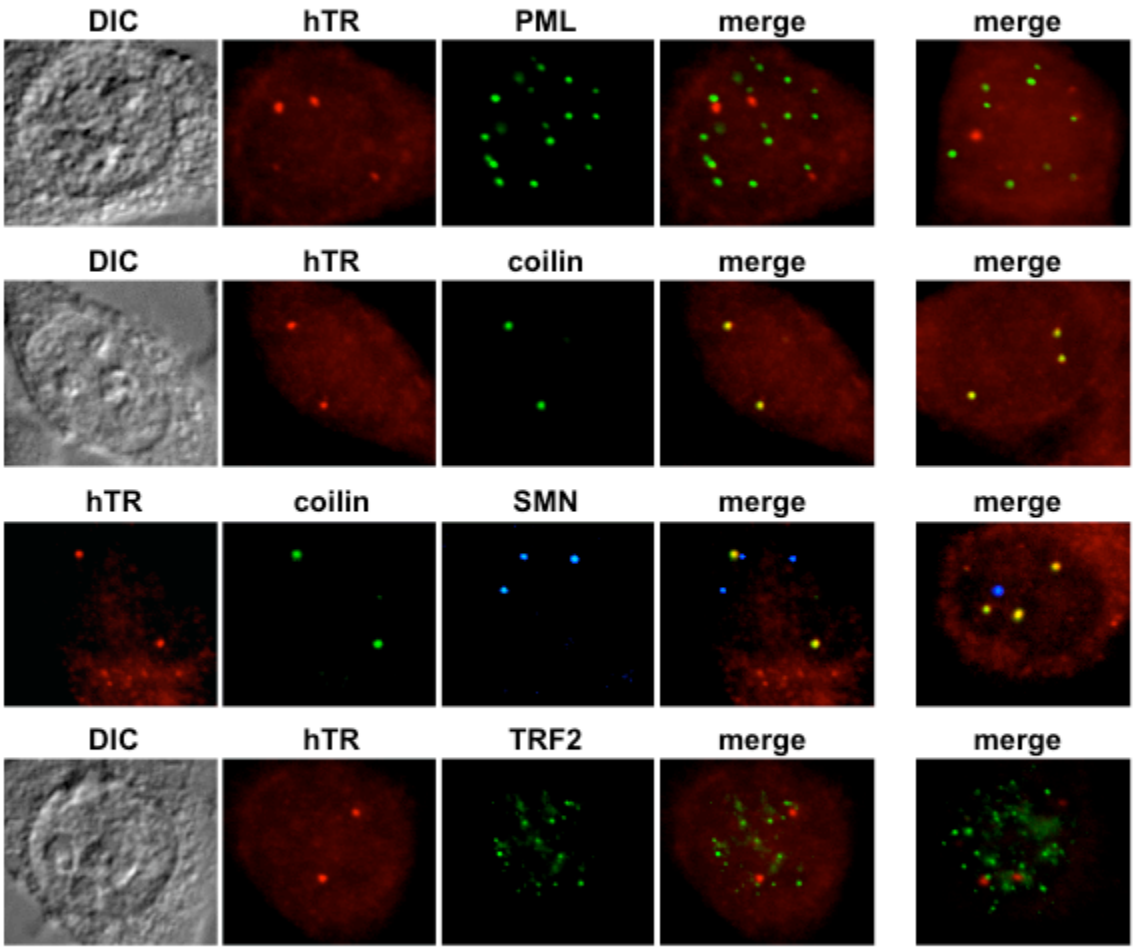

DIC
hTR
PML
merge
merge
DIC
hTR
coilin
merge
merge
hTR
coilin
SMN
merge
merge
DIC
hTR
TRF2
merge
merge

Figure A1.4. hTR is found in Cajal bodies in all telomerase-positive cancer cell lines examined. hTR FISH (red) was combined with IF with anticoilin antibodies (green) in MCF7, H1299, A549, and DU145 cells. Merged data from an additional cell is shown in the last column.

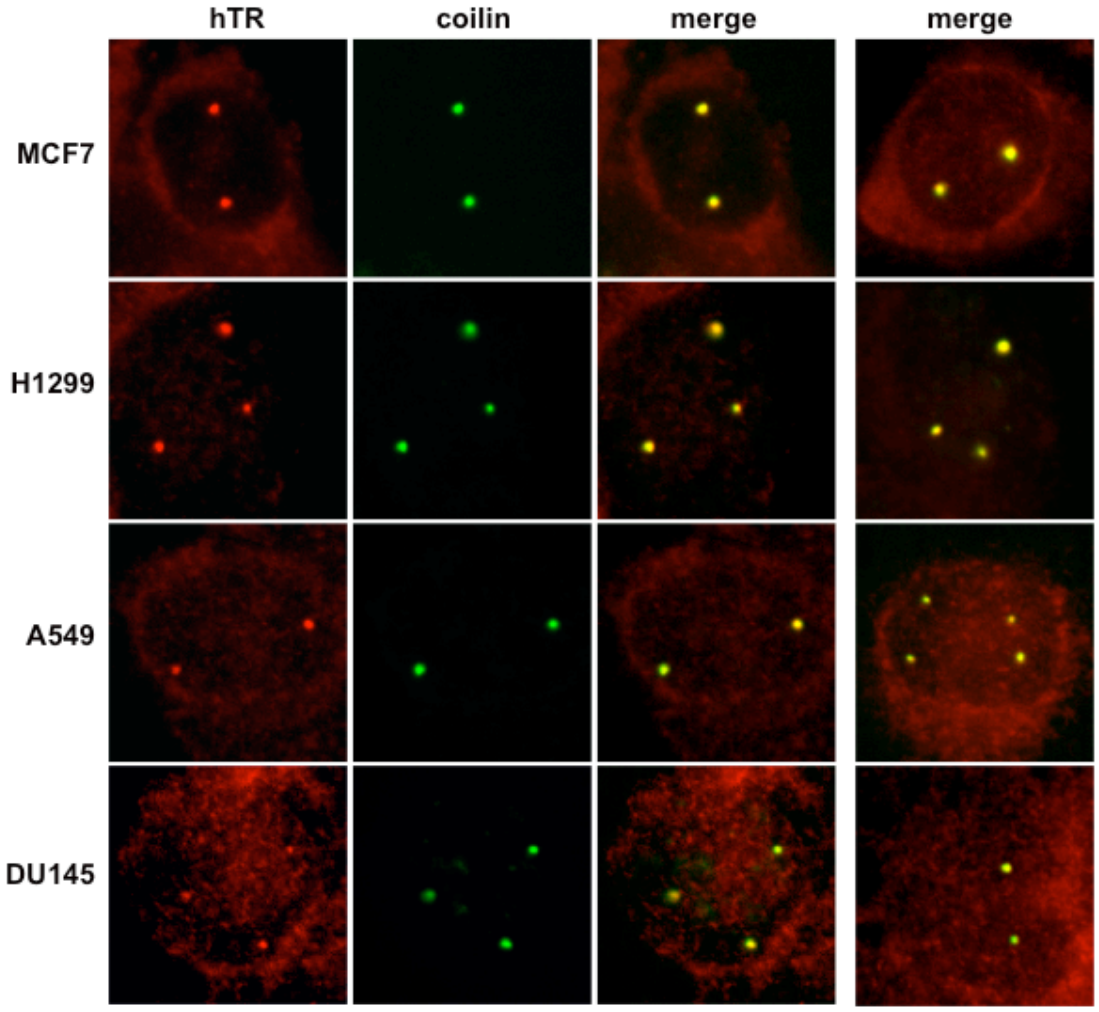

hTR
coilin
merge
merge
MCF7
H1299
A549
DU145

Figure A1.5. Ectopic expression of hTERT induces accumulation of hTR in nuclear foci including Cajal bodies. (A) hTR was examined by FISH (red) in BJ and BJ-TERT (BJ cells that stably expresses hTERT) cells. Arrows indicate obvious foci of hTR accumulation. (B) hTR FISH (red) was combined with IF with anticoilin antibodies (green) in BJ-TERT cells. Merge panel shows merged FISH and IF data. Arrow indicates a prominent Cajal body in all three panels. (C) hTR FISH (red) was combined with IF with anticoilin antibodies (green) in hTERT- and telomerase-negative U2OS cells. Merge panel shows merged FISH and IF data.

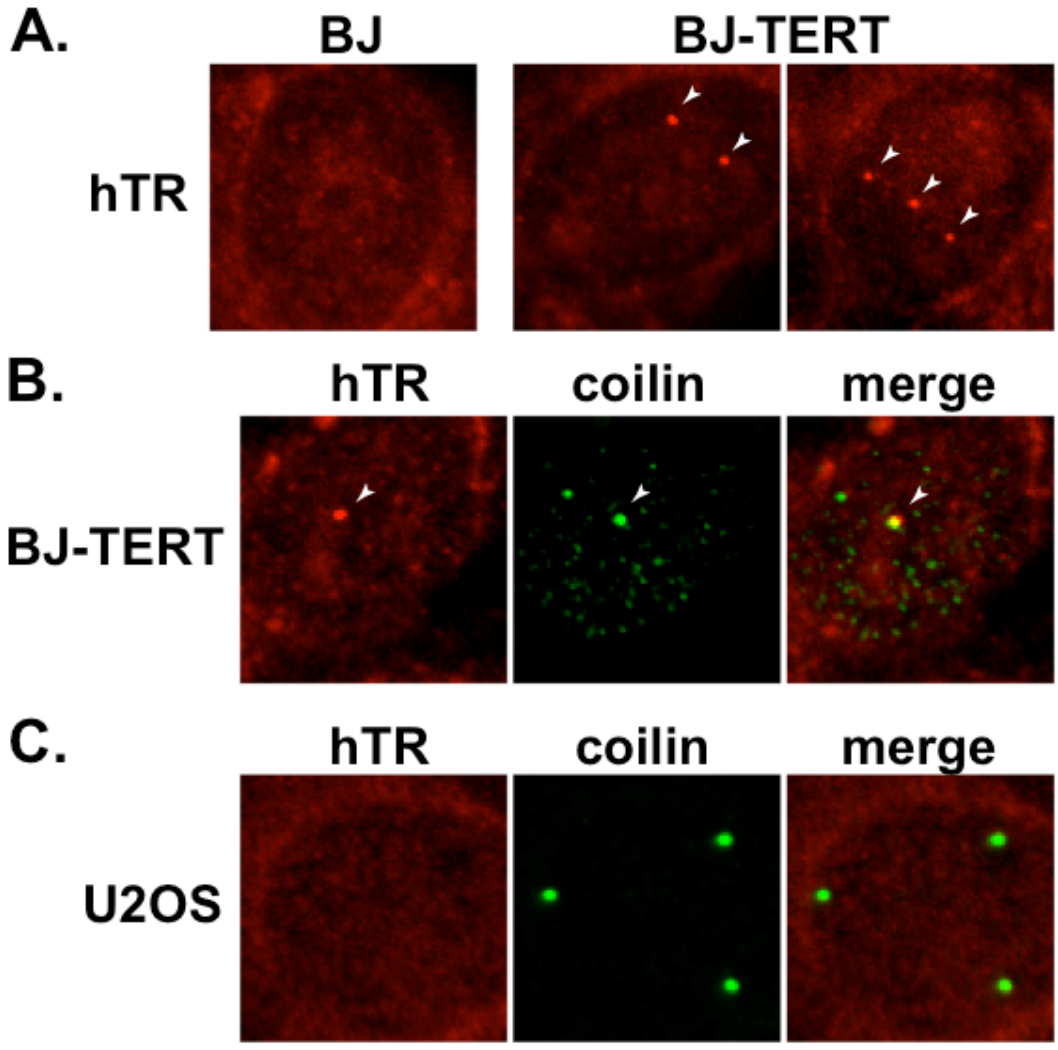
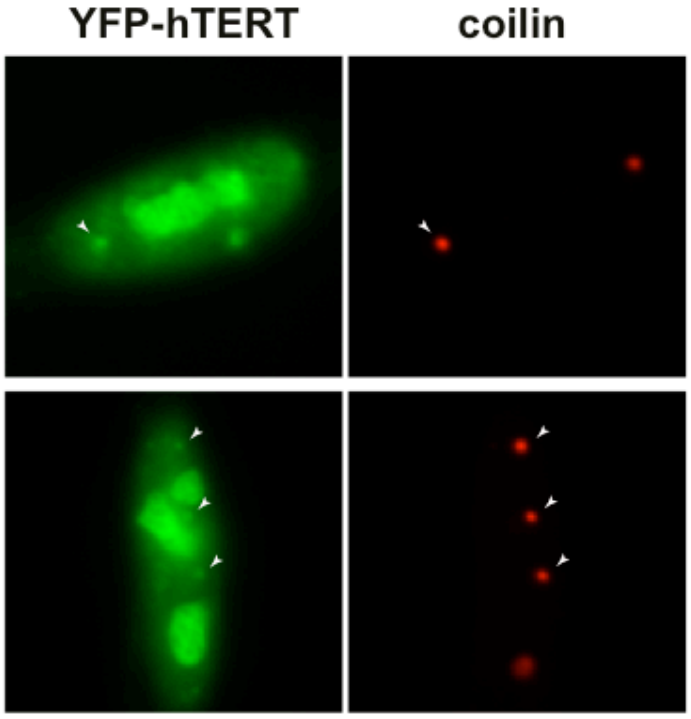

A.
BJ
BJ-TERT
hTR
B.
hTR
coilin
merge
BJ-TERT
C.
hTR
coilin
merge
U2OS

Figure A1.6. hTERT is present in Cajal bodies. YFP-TERT was transiently expressed in HeLa cells. Before fixation, cells were preextracted to remove soluble nucleoplasmic YFP-TERT. YFP-TERT (green) localizes to nucleoli and Cajal bodies (red, coilin, indicated in both panels by arrows).


YFP-hTERT
coilin

Appendix 2

Temporal Analysis of Telomerase Trafficking and Assembly in *Xenopus* Oocytes[1]

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[1]Zhu-Hong Li, Rebecca L. Tomlinson, Rebecca M. Terns, and Michael P. Terns, to be submitted to the *Journal of Cell Science*.

Abstract

Telomerase maintains telomere length and is strongly implicated in cancer cell immortalization in humans. The enzyme contains two essential subunits: telomerase RNA (TR) and telomerase reverse transcriptase (TERT). In vertebrate cells, TR and TERT are found at Cajal bodies and nucleoli as well as at telomeres. In order to better understand the intranuclear trafficking of telomerase, we examined the time course of both localization of TR and assembly of TR into telomerase complexes in the *Xenopus* oocyte system. We find that the major trafficking pathway for microinjected TR is through Cajal bodies into the nucleoplasm, with a small fraction of TR found in nucleoli at later time points. Localization of TR to nucleoli is not required for assembly of the enzyme. Assembly of TR with TERT is coincident with the localization of TR to Cajal bodies, suggesting that telomerase is assembled within this nuclear structure.

Introduction

Telomerase is the ribonucleoprotein (RNP) enzyme that functions to maintain telomere length in eukaryotic organisms (1). In humans, telomere maintenance and telomerase activation are critical steps in cellular immortalization and tumor progression, and the telomerase enzyme is well established as a target for development of cancer therapeutics (2).

Telomerase RNA (TR) and telomerase reverse transcriptase (TERT) are the core components of telomerase. *In vitro* reconstitution experiments have shown that TR and TERT are sufficient for catalytic activity, indicating that these are the minimal components of telomerase (3-5). TERT is a specialized reverse transcriptase that binds

TR directly and utilizes a short region in the RNA as a template for telomeric DNA repeat synthesis (6, 7). In general, eukaryotic organisms share highly conserved TERT proteins that interact with phylogenetically diverse TR molecules (8-10). Analysis of sequences of TRs from a wide range of eukaryotic species has revealed the evolution of at least three TR classes: ciliate, yeast and vertebrate (10-13). TRs from all studied organisms share certain essential features (including template sequences and pseudoknot domains), but in addition, appear to possess class-specific RNA motifs. For example, yeast TRs harbor a domain termed the Sm site, which is also found in small nuclear (sn)RNAs that function in pre-mRNA splicing (12, 14). In contrast, vertebrate TRs contain H/ACA and Cajal body (CAB) motifs, which are characteristic of the small nucleolar (sno) and small Cajal body (sca) RNAs that function in pre-ribosomal RNA and snRNA maturation, respectively (11, 15-19). Ciliate telomerase RNAs do not possess recognizable Sm sites, or H/ACA or CAB motifs (10). The Sm site and H/ACA and CAB motifs of yeast and vertebrate TRs are recognized by distinct sets of known proteins (14, 20-23) and appear to provide metabolic stability and nuclear localization to TR (18, 24-26). The proteins that bind these domains are not required for telomerase activity *in vitro*, but are likely essential for full activity in the cell.

It is clear that following their initial synthesis, TR and TERT must be assembled together to form a functional enzyme. However, we are only beginning to understand the pathways by which vertebrate telomerase is transported, assembled, and regulated. Recent studies implicate two intranuclear structures in telomerase trafficking: nucleoli and Cajal bodies.

The first clear evidence that TR is found in Cajal bodies and nucleoli came from *Xenopus* oocytes, where it was found that microinjected TR localizes to both structures (25, 27). The localization of TR to nucleoli is dependent on the H/ACA motif (25), which is also responsible for the nucleolar localization of snoRNAs (27). In human cells, the localization of endogenous TR has been examined by fluorescence *in situ* hybridization (FISH) (16, 28-30). In human cancer cells (but not primary cells) TR accumulates in Cajal bodies in interphase cells (16, 28-30). Specifically in S phase of the cell cycle, TR exhibits dynamic changes in localization in telomerase-positive human cancer cells (29, 30). During early S phase, a fraction of endogenous TR localizes to the surface of nucleoli (29). Subsequently, during mid-S phase, when telomere synthesis likely occurs, TR appears in foci associated with Cajal bodies and at subsets of telomeres ([29], but see also [30]). The localization of TR to both Cajal bodies and telomeres is dependent upon expression of TERT (Chapter 3).

TERT has also been found associated with both nucleoli and Cajal bodies. Ectopically expressed TERT exhibits a prominent nucleolar localization in mammalian cells (31-33) and a less obvious association with Cajal bodies (28). However, examination of endogenous TERT by immunofluorescence analysis reveals a significantly different pattern. TERT is found in intranuclear foci not associated with nucleoli or Cajal bodies during most of the cell cycle in cancer cells (29). In early S phase, a fraction of TERT moves into the nucleolus, and in mid-S phase TERT (like TR) is found in Cajal body-associated foci and at telomeres (29). The association of TERT with telomeres (and Cajal bodies) can be enhanced by expression of hTR (Chapter 3).

The pattern of TR and TERT localization over the course of the cell cycle in cancer cells provides tantalizing suggestions about the regulation of telomerase in these cells. It is tempting to hypothesize that the majority of TR and TERT are sequestered in separate locations throughout most of the cell cycle and assembled for function in S phase at Cajal body-associated foci or telomeres. However, because some telomerase activity can be detected throughout the cell cycle ([34] but see also [35]), it is not clear how (or if) the localization relates to assembly. Moreover, these studies do not address the initial pathway traveled by nascent TR molecules following synthesis.

In this study, we have used the *Xenopus* oocyte system to investigate the time course of both the localization of xTR and assembly of telomerase following microinjection. The oocyte is unique in its capacity to provide this kind of dual analysis of newly introduced molecules (36). Our results indicate that telomerase RNA is rapidly assembled into functional telomerase complexes following microinjection into *Xenopus* oocyte nuclei. Detection of catalytic activity correlates with localization of TR to Cajal bodies and precedes nucleolar localization. Furthermore, we have found that the assembly of xTR and TERT does not require nucleolar localization, as mutation of the H/ACA motif of TR, slows, but does not prevent, its assembly into an active enzyme *in vivo*. Subsequent mutation of the CAB box of xTR does not affect assembly of the injected RNA into active telomerase enzyme, but also does not prevent the initial localization of TR to Cajal bodies. Taken together, our results suggest that following synthesis, TR molecules are rapidly transported to Cajal bodies, where they are assembled with TERT to form telomerase complexes. The assembled molecules are

then transported to the nucleoplasm, where the functional enzyme resides in *Xenopus* oocytes.

Results

Telomerase RNA assembles into a functional enzyme following injection into Xenopus oocytes.

Previously, we have shown that telomerase RNA is retained within the nucleus and associates with both Cajal bodies and nucleoli following injection into *Xenopus* oocyte nuclei (25, 27). Here we tested whether micro-injected Xenopus telomerase RNA (xTR) is incorporated into functional telomerase complexes in oocytes (Figure A2.1). Telomerase assembly was determined using a standard, PCR-based TRAP (telomeric repeat amplification protocol) assay that measures addition of telomeric repeats (by assembled telomerase) to a defined oligonucleotide primer *in vitro* (37). Telomerase activity is detected as a ladder of products incrementally larger than the primer. To specifically assay the activity of the injected telomerase RNA (and not the activity of endogenous telomerase), we altered the template region (see Figure A2.1A) of *Xenopus* telomerase RNA (AATCCC to AAACCC) and used reaction conditions and primers specific for the unique telomeric repeats that would be synthesized by the altered template TR (TTTGGG rather than TTAGGG) (38). Using this modified set of conditions, the activity of endogenous (wildtype template) telomerase is not detected, even when an equivalent amount of wildtype template TR is injected (Figure A2.1B, lanes 1 and 2). Activity is detected following injection of the altered template TR (Figure A2.1B, lane 3), and is sensitive to RNAse treatment (Figure A2.1B, lane 4). The

resulting TRAP assay products were sequenced to verify that the products were derived from the injected, altered template TR (data not shown). In addition, we performed control experiments to ensure that the telomerase activity that we detect in injected oocytes reflects enzyme assembled *in vivo* and not *in vitro* under the TRAP assay conditions. Mixing the altered template xTR with (uninjected) nuclear extract does not result in assembly of the RNA into an active enzyme *in vitro* (Figure A2.1C, lane 2). In addition, the activity of the altered template xTR was not affected by the addition of wildtype competitor RNA *in vitro* (data not shown). Therefore, the telomerase activity that we detect represents the assembly of exogenous TR with endogenous TERT *in vivo* (Figure A2.1C, lane 3). Thus, the assay is specific for activity of the injected TR, and moreover, the injected TR assembles with TERT in the oocyte to form functional telomerase complexes.

Injected telomerase RNA moves rapidly through Cajal bodies into the nucleoplasm.

Our finding that injected xTR assembles into functional telomerase complexes allowed us to address when and where the RNA assembles with TERT by examining the time course of both movement (Figures A2.2 and A2.3) and activity (Figure A2.4) of the injected molecules. The general strategy is illustrated in Figure A2.2A. Nuclei were isolated from oocytes over an extensive time course following injection of labeled TR into nuclei. At each time point we assayed the following: 1) the percentage of injected xTR in intranuclear structures vs. nucleoplasm (via biochemical fractionation), 2) localization of the injected RNA relative to specific nuclear structures (via microscopy of

nuclear spreads), and 3) the extent to which the injected RNA had assembled into a functional RNP (via the modified TRAP assay described above).

To directly assess the amount of xTR found in the nucleoplasm and nuclear structures as a function of time following injection, we micro-injected radiolabeled xTR into *Xenopus* oocyte nuclei, and, at various time points, fractionated the nucleus into structures and nucleoplasm (by centrifugation). Over a 72 hour time course, we followed the distribution of xTR and two co-injected control RNAs (U3 snoRNA and U1 snRNA). As expected, U3 snoRNA moved gradually from the nucleoplasm to structures (Cajal bodies and later nucleoli, see [39]), while the majority of U1 snRNA was found in the nucleoplasm at all time points (Figure A2.2B and A2.2C). We found that xTR undergoes a rapid association with nuclear structures and then eventually moves to the nucleoplasm (Figure A2.2B and A2.2C). At the earliest time point that we examined, 30 minutes after injection, most of the xTR (but very little U3 or U1 RNA) was found in the pelleted structures. By ~8 hours after injection most of the xTR was in the nucleoplasmic fraction, where it eventually appeared to reach a steady state level of 70-80%, similar to that observed for the endogenous xTR (25). Similar temporal profiles were observed using injected RNA amounts ranging between 0.01 and 1.0 fmol of xTR (data not shown).

To determine which structures the RNA is associated with at the various points in the time course, we followed fluorescently labeled xTRs via microscopy (Figure A2.3). Control experiments clearly demonstrated that our method of labeling xTR with fluorescein (by transcription with fluorescein-12-UTP) does not significantly alter any of the biological properties of the RNA that we examined, which included stability, nuclear

retention, biochemical fractionation, and assembly into functional telomerase (this work, [25, 39]). We had previously found that xTR localized to both Cajal bodies and nucleoli using this approach (25). Technical improvements have allowed us to inject less RNA (1 fmol instead of 3 fmols) than in the earlier work, providing better resolution in the time course. At early time points (30 min, Figure A2.4, and also 15 min, data not shown) xTR is strongly associated with Cajal bodies (immunostained with antibodies against the Cajal body marker protein coilin and indicated by arrows). No signal was observed in nucleoli (the larger structures visible in the fields) at the early time points. At later time points, the signal in Cajal bodies decreases and thereafter remains at a low level that is nevertheless significantly above background signals seen in uninjected oocytes (see Figure A2.4, top set of panels). At later time points (24 to 48 hours) we also observed localization of the RNA to nucleoli. These results indicate that the majority of xTR moves through Cajal bodies at early time points following injection. A small fraction of xTR remains stably in Cajal bodies throughout the time course and appears in nucleoli at later time points.

The temporal pattern of xTR localization in nuclear structures (Figure A2.3) is in good agreement with the results of the biochemical fractionation experiments (Figure A2.2). The early and transient association of the majority of xTR with nuclear structures (Figure A2.2) appears to be attributable to localization to Cajal bodies (Figure A2.3). At later time points the small fraction of xTR associated with structures (Figure A2.2) can be found in both Cajal bodies and nucleoli (Figure A2.3).

Assembly of telomerase is coincident with localization of xTR to Cajal bodies (and precedes localization to nucleoli).

A question of great interest is when and where in the cell telomerase is assembled into an active enzyme. Using the *Xenopus* oocyte system, we can assess the assembly of the injected (altered template) xTR with TERT by the modified TRAP assay (Figure A2.1) over time and relate the timing of assembly to the location of the RNA. Figure A2.4A shows the telomerase activity associated with the injected xTR over an extensive time course. As our results clearly show, there is significant telomerase activity as early as 15 minutes after injection, indicating that the RNA is quickly assembled with TERT into a functional complex. The kinetics of telomerase assembly (early) correspond to the timing of Cajal body localization, but are incongruent with the kinetics of nucleolar localization (late) (see Figure A2.3).

The coincidence of assembly of TR and TERT with the transit of xTR through the Cajal body suggests a model in which xTR is assembled with TERT in the Cajal body and then the newly made complex moves to the nucleoplasm. To test this idea more directly, we next examined telomerase activity in the nucleoplasm and nuclear structures over the time course. Following injection of the altered template xTR, oocyte nuclei were fractionated into structures and nucleoplasm (by centrifugation) at various time points and modified TRAP assays were performed. We found that the telomerase complex formed by the injected xTR overwhelmingly resides in the nucleoplasm (supernatant) at all time points examined (Figure A2.4A). In addition, we consistently observed low levels of telomerase activity in the nuclear structure fraction (pellet) at early time points (30 min to 8 hours) after injection, but not at later time points (Figure

A2.4A). These results are also consistent with the suggestion that telomerase assembly occurs in Cajal bodies and that the complexes rapidly exit to the nucleoplasm following assembly.

Analysis of the localization of endogenous telomerase indicated that essentially all steady-state activity was observed in the nucleoplasmic fraction (Figure A2.4B, upper panel). No activity was detected in association with the small fraction of endogenous xTR found in nuclear structures at steady-state (Figure A2.4B, lower panel). Together, these results indicate that active telomerase resides in the nucleoplasm of *Xenopus* oocytes and that the enzyme assembled from the injected telomerase RNA reaches a similar steady-state distribution as endogenous telomerase.

Nucleolar localization and the H/ACA motif of telomerase RNA are not essential for telomerase RNP formation in vivo.

To further address if localization of telomerase RNA to either the nucleolus or Cajal body is required for telomerase assembly, we made mutations in elements (the Box H/ACA motif or the CAB box) of telomerase RNA that are known to be involved in localization to these sites (16, 25). The box H/ACA motif was first recognized in small nucleolar RNAs and is responsible for targeting these RNAs to the nucleolus (27, 40). We had previously shown that mutations in the H/ACA motif (box H or box ACA, see Figure A2.2A) prevent localization of telomerase RNA to nucleoli (25). In order to specifically address whether nucleolar localization is essential for telomerase assembly, we tested the effect of box H and box ACA mutations on the ability of the altered template xTR to assemble with TERT into a functional complex. We found that both H/ACA motif mutants formed functional complexes (Figure A2.5A, compare H and ACA

to uninjected). However, the level of activity found with the H/ACA mutants was less than that observed with the wildtype RNA, particularly at early time points (Figure A2.5A, compare H and ACA to WT at 1 hour and 3 hours). Mutations in the H/ACA motif greatly reduce the nuclear retention of TR (resulting in loss of TR to the cytoplasm) and also decrease the stability of the RNA (25). Thus, the amounts of the H/ACA mutant RNAs present in the nucleus at the times when activity was assayed were significantly less than the amount of control wildtype RNA (Figure A2.5B). To better compare the RNAs, we assayed a range of wildtype xTR concentrations, identified samples that produced telomerase activity similar to the mutant (Figure A2.5C, arrows), and examined the corresponding amounts of RNA present in the nucleus at the time of assay (Figure A2.5C, lower panel). We found that the amount of RNA required to produce equivalent levels of telomerase activity was ~10 fold more mutant than wildtype xTR at 1 hour after injection, but only ~3 fold more mutant than wildtype xTR at 3 hours, indicating that the H/ACA mutant RNA assembles more slowly. As we had previously reported, we do not detect localization of the H/ACA mutants in nucleoli (25); however, like wildtype xTR, the H/ACA mutants do associate with Cajal bodies (Figure A2.5D). Thus, nucleolar localization of xTR is not essential for assembly of a functional TR/TERT complex, and the ability to form functional telomerase complexes *in vivo* does not require an intact H/ACA motif.

The CAB box of Telomerase RNA is not essential for Cajal body targeting and telomerase assembly.

In human cells, mutation of the CAB box results in a change in the site of the steady-state accumulation of telomerase RNA from Cajal bodies to nucleoli (16). We

mutated the CAB box with the expectation that the mutation would prevent localization of xTR to Cajal bodies and allow us to assess the importance of Cajal body localization in telomerase assembly. We found that the CAB box is not essential for telomerase RNA stability, nuclear retention, or assembly (Figure A2.6A and data not shown). The same level of activity was observed with both the injected CAB box mutant and wildtype RNA (Figure A2.6A), and is consistent with previous findings in human cells (23). However, the CAB box mutation did not prevent Cajal body localization; like wildtype xTR, the CAB box mutant is strongly associated with Cajal bodies at early time points (Figure A2.6C, compare mutant and wildtype TR profiles in left panels). While the CAB mutation did not stop Cajal body localization of xTR, it significantly reduced the time of association of the RNA with Cajal bodies (as well as the nucleoplasm) and also increased both the speed and strength of association of the RNA with nucleoli (Figure A2.6B and A2.6C, compare mutant and wildtype TR profiles). The late hour xTR CAB mutant localization profile is consistent with the previous localization study in human cells (16). In addition, the technical advantages of the *Xenopus* system allowed us to determine that there is a transient association of xTR with Cajal bodies at early time points even when the CAB box is mutated. Although this particular experimental approach did not permit us to test whether Cajal body localization is essential for telomerase assembly, the results are still in good agreement with the hypothesis that telomerase is assembled in Cajal bodies. Moreover, our data suggest that the CAB box may be involved in Cajal body retention (or prevention of nucleolar localization) rather than in Cajal body targeting.

Discussion

Understanding how the telomerase enzyme is assembled *in vivo* from its component parts is critical toward understanding telomerase function and regulation and may contribute to efforts aimed at combating cancer by interfering with telomerase function (2). All vertebrate cells contain telomerase complexes composed of structurally related telomerase RNA and reverse transcriptase molecules that add TTAGGG repeats to chromosome termini (11, 41). Likewise, conserved biogenesis strategies are very likely employed in different vertebrate cell types. Recent studies from our lab and others indicated that telomerase trafficking is a cell cycle dependent, well-regulated process (29, 30). However, these studies only assess localization of telomerase components, and do not directly address questions of telomerase biogenesis, namely where within the cell assembly occurs. The work described here demonstrates that *Xenopus* oocytes offer a powerful *in vivo* system for the analysis of vertebrate telomerase biogenesis and trafficking. By following the intranuclear distribution, localization, and activity of wildtype and mutant xTR molecules as a function of time spent in living oocytes, we have gained detailed information regarding the pathway traveled by telomerase RNA, including where and when the RNA is incorporated into a functional telomerase enzyme. Collectively, our findings implicate Cajal bodies as conserved and important sites of telomerase biogenesis.

Cajal Bodies and the Biogenesis of Telomerase

Together with previous findings in mammalian cells ([16, 28] and Chapter 3), our results point to the Cajal body as a conserved site along the pathway of vertebrate telomerase biogenesis and function. Cajal bodies are spherical intranuclear bodies

discovered ~100 years ago in neurons by the Spanish scientist Ramon y Cajal (42) that have since been shown to be common to plant and animal cells (43). A number of recent findings have provided a collective view that Cajal bodies serve as centers of post-transcriptional RNA modification (44, 45) and RNP assembly for a variety of nuclear complexes (46-48).

A multitude of possible telomerase biogenesis steps can be envisioned to occur within Cajal bodies consistent with the known or proposed functions of this nuclear structure. For example, given that several small nuclear RNAs appear to undergo their posttranscriptional modification within Cajal bodies (44), it follows that Cajal bodies may be the site where telomerase RNA is modified (by the 5' cap hypermethylase (16) or predicted scaRNAs that guide putative site-specific TR nucleotide modifications or although, to date, no such sites of modification have been mapped for TR). In addition, several lines of evidence indicate that telomerase assembly occurs at Cajal bodies. For example, the finding that both telomerase RNA (Figures A2.3, A2.5, A2.6 and [16, 25, 28]) and several telomerase protein components including TERT (the catalytic subunit of telomerase) and proteins that recognize the box H/ACA domain of telomerase RNA localize to Cajal bodies (21, 28) is consistent with the proposal that these components are joined into telomerase RNP complexes in this nuclear body. Moreover, the assembly of telomerase RNA into active telomerase enzyme in *Xenopus* oocytes occurs with comparable kinetics as the association of the RNA with Cajal bodies (Figures A2.3-A2.5). In mammalian cells the accumulation of telomerase RNA at Cajal bodies correlates with capacity of the cells to assemble telomerase enzyme. Specifically, telomerase RNA accumulates in the Cajal bodies of a variety of cancer cell lines (where

telomerase assembly is ongoing) but not primary cell lines (where little to no telomerase is being made) (16, 28). Ectopic expression of hTERT in primary cells reinstates telomerase assembly and leads to accumulation of telomerase RNA in Cajal bodies and knockdown of hTERT expression in cancer cells (by RNAi) leads to a loss of telomerase RNA from Cajal bodies (Chapter 3 and [28]). Finally, recent data also suggests that the assembly of telomerase may be mediated by a chaperone of RNP assembly that selectively localizes to Cajal bodies within the nucleus, called the survival of motor neuron (SMN) complex (47, 49). A physical association between the SMN complex (consisting of the SMN protein and six additional gemin proteins) and telomerase has been observed; the association is apparently mediated via direct interaction of the SMN protein with two different telomerase components (TERT and GAR1) (50, 51). While our data does not exclude the possibility that one or more telomerase assembly steps occur in the nucleoplasm (prior to or immediately after TR traverses Cajal bodies), the available information is most consistent with a model where initial telomerase assembly occurs within Cajal bodies followed by a rapid export of the assembled complexes to the nucleoplasm (where they are presumably available to encounter telomeres for function).

A Role for Nucleoli in Telomerase Biogenesis?

The findings that both telomerase RNA (18, 25) and TERT (29, 31-33) are detected at nucleoli (at least in specific cell types under certain conditions) have led to the idea that telomerase assembly takes place within nucleoli. However, our results suggest that the nucleolus is not a required site where TR and TERT meet to form an active telomerase enzyme. We have found that detection of telomerase catalytic activity precedes the appearance of injected telomerase RNA in nucleoli by a number of

hours (and is coincident with appearance of the RNA in Cajal bodies) (Figures A2.3 and A2.4). Furthermore, disruption of the Box H/ACA motif, which prevents the nucleolar localization of TR (25), does not prevent TR association with either Cajal bodies or xTERT *in vivo* (Figure A2.5). Finally, the activity of injected telomerase RNA was detected in pelleted nuclear structures only at early times (when TR is in Cajal bodies) but not late times (when TR is detected in nucleoli) (Figure A2.4B).

We show that little or no endogenous telomerase RNA is present in nucleoli of Xenopus oocytes under steady state conditions (Figure A2.4B) but that a small fraction of injected telomerase RNA is targeted to nucleoli at very late times after injection (Figures A2.3 and A2.4). While it is formally possible that the nucleolar localization of injected TR reflects a late step in telomerase biogenesis (e.g. TR modification or association of regulatory protein[s]), it is also possible that TR may associate with nucleoli only when TR levels are in excess of that which can be accommodated by factors of the telomerase biogenesis pathway. Consistent with this possibility, telomerase RNA appears at oocyte nucleoli at early time points if the levels of injected telomerase RNA are increased (25). Similarly, ectopic overexpression of tagged TERT in human cells leads to the localization of the protein in nucleoli (31-33) (but endogenous hTERT primarily resides in nuclear foci distinct from nucleoli [29]). Biochemical fractionation data also provide support that the vast majority of telomerase RNA does not reside in nucleoli in human cells (18).

While the data presented here suggests that the nucleolus may not be an essential, conserved site for TR/TERT interaction, nucleoli may be important for cell cycle dependent steps of telomerase holoenzyme formation or in regulating telomerase

activity and recruitment to telomeres. The nucleolus is now known to orchestrate a number of cellular functions beyond ribosome biogenesis including cell cycle regulation, viral replication, regulation of cellular survival and proliferation (52-54). Analysis of the nucleolar proteome has revealed dynamic associations of specific proteins with nucleoli in response to changes in metabolism or at specific phases of the cell cycle (55). Our own work has shown both endogenous TR and TERT transit to nucleoli in a cell cycle-dependent manner that precedes localization of the enzyme components to telomeres (29). Furthermore, localization studies with ectopically expressed hTERT suggest active telomerase may be targeted to nucleoli as part of a regulatory mechanism that limits access of the enzyme to telomeres only during times of telomere elongation and replication (32). The model is based on the observation that the intranuclear distribution of ectopically expressed TERT changes between the nucleolus and the nucleoplasm in response to cell cycle phase, expression of viral oncogenes, and ionization-induced DNA double-stranded breaks (32).

Role of the Box H/ACA motif and CAB box in telomerase RNA trafficking, telomerase biogenesis and function.

The discovery that all vertebrate telomerase RNAs contain a conserved RNA domain known as the Box H/ACA motif, previously found in (and required for the nucleolar localization of) snoRNAs, suggested that telomerase RNA might exhibit the localization properties of snoRNAs (i.e. predominantly nucleolar with a minor fraction localizing to Cajal bodies). More recently, the superfamily of Box H/ACA RNAs expanded with the discovery of a new class of RNAs, called scaRNAs, which appear to stably reside at Cajal bodies (exhibiting a similar pattern to the localization of TR [16,

28]), where they modify spliceosomal RNAs (15). These scaRNAs contain a sequence motif known as the Cajal body box (CAB) (consensus; UGAG) which is located in the CR7 domain of TR [16, 23]).

Functional assays of telomerase activity have shown that conserved sequences found at the extreme 3' end of the TR molecule (including the H/ACA domain and CR7 domain that harbors the CAB box) are non-essential *in vitro* (3, 56-58). On the other hand, we and others (18, 25, 26) have demonstrated the essential nature of TR sequences such as the H/ACA domain for telomerase activity *in vivo*. The H/ACA domain of hTR is responsible for keeping the RNA within the nucleus (the functionally relevant cellular compartment) and is required for targeting the RNA to nucleoli (25). Together with the adjacent CR7 domain, the H/ACA domain is also important for hTR processing and accumulation (26). In this work, we show that while an intact H/ACA domain is not essential for Cajal body targeting or telomerase assembly *in vivo*, the rate of telomerase assembly is reduced by Box H/ACA mutations. This observation suggests that localization to nucleoli is not essential for TR/TERT interaction. Moreover, these findings suggest that proteins which recognize the H/ACA motif (i.e. dyskerin, GAR1, Nhp2, and Nop10) may help recruit or stabilize TERT binding *in vivo* (or that the H/ACA motif helps stablize the RNA into a conformation that more favorably binds TERT).

The steady state (late hour time point) localization profile of CAB mutated xTR is in good agreement with that of CAB mutated hTR in human cells (ie, localization of the CAB mutated RNA to nucleoli instead of Cajal bodies and nucleoplasm). The unique features of the *Xenopus* oocyte system allowed us to successfully detect the strong

Cajal body association of CAB mutants at early hour time points which can not be visually observed in the mammalian cell culture system. Our data indicates that the CAB box is not essential for TR/TERT interaction or targeting of the RNA to Cajal bodies. Instead, our result suggests that the CAB box influences Cajal body localization by promoting retention of TR in Cajal bodies or preventing the RNA from trapping inside of nucleoli (Figure A2.6). Likewise, in human cells mutation of the CAB box does not affect assembly with TERT or cap hypermethylation of TR (a maturation step presumed to occur within Cajal bodies) (16, 23). Box H/ACA scaRNAs typically contain two CAB boxes, while (to date) only one has been identified in TR (16, 59). Perhaps a second, as yet identified, CAB motif exists in TR that functions to direct xTR to Cajal bodies, or the Cajal body association of the CAB mutant xTR could be directed by some other unknown cis element(s) and its binding proteins. It will be important to understand what the role that cis and trans-acting factors play in mediating the Cajal body targeting and retention of TR. Cajal bodies not only have important links to telomerase biogenesis (outlined above), but have also been implicated in delivery of telomerase to telomeres (16, 29). Despite the wealth of information garnered from the our use of the *Xenopus* oocyte system, more work needs to be done to precisely elucidate the role of Cajal bodies and nucleoli in telomerase biogenesis, trafficking and function.

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Materials and Methods

Constructs: Wild type xTR, U3, and U1 constructs were previously described (25). The altered template xTR (TTAGGG changed to TTTGGG) was generated by PCR using the wildtype xTR gene as the template and primers: 5'-GAATCAGCGTTTAAAGCTCAATGTGGACGGAGGTCTCTGTTTCGCAAACCCAAATACACTGGC-3' and 5'-ACATGTCGGGGACTGGCTG-3'. All other mutant telomerase RNAs were generated by PCR using the altered template xTR construct as the template. The box ACA xTR mutant (ACA changed to CCC) was generated using primers: 5'-ATTTAGGTGACACTATAGAATCAGCGTTTAAAGCTCAATG-3' and 5'-ACAGGGCGGGGACTGGCTGAAC-3', Box H xTR mutant (AGAAAA to UGUAUU) using primers: 5'-GTCGGGTGTATTTAGGGGGCGCGCTGGTG-3' and 5'-CCCCCTAAATACACCCGACCCCCCAGAC-3', and CAB xTR mutant (UUAG (nt) to UUUC) using primers: 5'-GTCATGCTTTCCCTTTCTTGTGG-3' and 5'-CCACAAGAAAGGGAAAGCATGAC-3'. All constructs were cloned and their sequences confirmed.

***In vitro* transcription:** *In vitro* transcription was performed using 100 ng of PCR product in a standard 10 µL reaction (25). Radiolabeled RNAs (used in biochemical fractionation) were transcribed by SP6 RNA polymerase in the presence of [α-^{32}P]GTP (800 Ci/mmol; ICN/MP Biomedicals, Costa Mesa, CA) while fluorescently labeled RNAs (used in nuclear spreads) were transcribed in the presence of [α-^{32}P]GTP and

fluorescein-12-UTP (Roche, Indianapolis, IN; used in a 1:1 ratio with UTP). For fluorescence xTR transcription, 2 mM m^7GpppG cap was used instead of the 500 μM used in our standard reaction.

Oocyte microinjection, nuclear spreads, and microscope analysis: RNAs were microinjected into stage V and VI *Xenopus* oocyte nuclei as described (39). Briefly, 10 nL solution containing *in vitro* transcribed RNAs were microinjected into each nucleus. Following injection, the oocytes were cultured at 18°C in 1X MBSH (88 mM NaCl, 1 mM KCl, 2.4mM $NaHCO_3$, 20 mM HEPES, pH 7.5, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, containing 10 μg/mL penicillin and streptomycin) throughout the time course. At different time points after injection, the cells were manually dissected and the nuclei were removed and used for either biochemical fractionation (see below), TRAP assays (also below), or nuclear spreads. Nuclear spreads were prepared essentially as described (25). To detect Cajal bodies, indirect immunofluorescence was performed using a polyclonal antibody against *Xenopus* p80-coilin (R31, 1:5000 dilution) and Texas red-conjugated anti-rabbit second antibody (1:100 dilution, Jackson Immunoresearch, West Grove, PA). Images were obtained on a Zeiss Axiovert S 100 inverted fluorescence microscope equipped with differential interference contrast (DIC) optics (Carl Zeiss, Thornwood, NY). All images were acquired at 63x magnification using a cooled charge-coupled device camera (Quantix-Photometrix, Tucson, AZ) and IP Lab Spectrum software (Signal Analytics, Fairfax, VA).

Biochemical fractionation and Northern blotting: At each time point at least 3-5 nuclei were transferred to 50-100 μL D250 solution (50 mM Tris-HCl, pH 7.6, 25 mM KCl, 10 mM $MgCl_2$, 250 mM Sucrose and 3 mM fresh DTT) on ice, and the nuclear

membranes were destroyed by pipetting 20 times with a 200 µL pipette. The nuclear contents were then fractionated at 20,000 x g for 15 min at 4 °C. After the supernatant was transferred to another tube, the pellet was resuspended in an equivalent volume of D250. The RNAs in each fraction were then extracted using standard methods and separated by denaturing PAGE. The gels were dried and exposed to a phosphorimager screen. A Northern blot to detect endogenous xTR distribution (Figure A2.4B) was performed using methods and radiolabeled probes as described previously (25). All data was visualized using a Phosphorimager (Amersham/GE Healthcare, Little Chalfont, Buckinghamshire, UK) and quantitated using ImageQuant software (Molecular Dynamics/GE Healthcare).

Telomerase extraction and activity assay: Nuclei were isolated and directly transferred into CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol). The nuclear membrane was disrupted by repeated pipetting. The solution was placed on ice for 30 minutes and then centrifuged at 10,000 x g for 10 mins at 4°C. The supernatant was transferred to another tube, frozen and kept at –80°C for future use. When TRAP assays followed biochemical fractionation, equal amount of solutions from pellet or supernatant were transferred to lysis buffer and incubated on ice, using the same conditions performed on total nuclei. For detection of endogenous telomerase activity (Figure A2.4B), the TRAPeze Telomerase detection kit (Chemicon/Millipore, Temecula, CA) was used. To detect activity from the injected altered template xTR, the TRAP assay was modified as in (38). The extension of altered template telomerase was performed at 30°C for 30 min to 1hr in 15 µL total volume in the absence of dATP and

dCTP nucleotides. The lysate was heat treated to eliminate the telomerase activity and 1-5 µL extension product was used for PCR amplification using a primer specific for these extension products (5'-CCGCGAAACCCAAACCCAAACCCA-3') instead of the wild type comeback primer provided by the kit.

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Figure A2.1. Injected xTR assembles into active telomerase enzyme in *Xenopus* oocytes. *A. The secondary structure of xTR.* Structure modified from (11). Substitution mutations were made in the template region, and the Box H, Box ACA and Cajal Body Box (CAB) of the Box H/ACA sno/scaRNA motif. *B. Injected xTR associates with endogenous xTERT to form active enzyme.* Altered template xTR was injected into oocytes and a modified TRAP assay specific to this injected xTR was performed. Internal control for PCR reaction is indicated (IC). Activity (ladder of bands above IC) is observed upon injection of this xTR (Alt. template) and is lost with RNAse A treatment (RNAse). Using the modified TRAP assay, activity was not detected in uninjected cells (uninjected) or in cells injected with xTR containing a wildtype template (WT). *C. Assembly occurs in vivo, not in vitro.* Altered template xTR was injected into *Xenopus* oocytes and the nuclei were dissected 1 hour after injection and transferred to lysis buffer (*in vivo*). The same amount of RNA was added into lysis buffer and incubated on ice for 30 minutes with uninjected nuclei (*in vitro*). The modified TRAP assay was performed on these lysates and uninjected control lysate (uninjected).

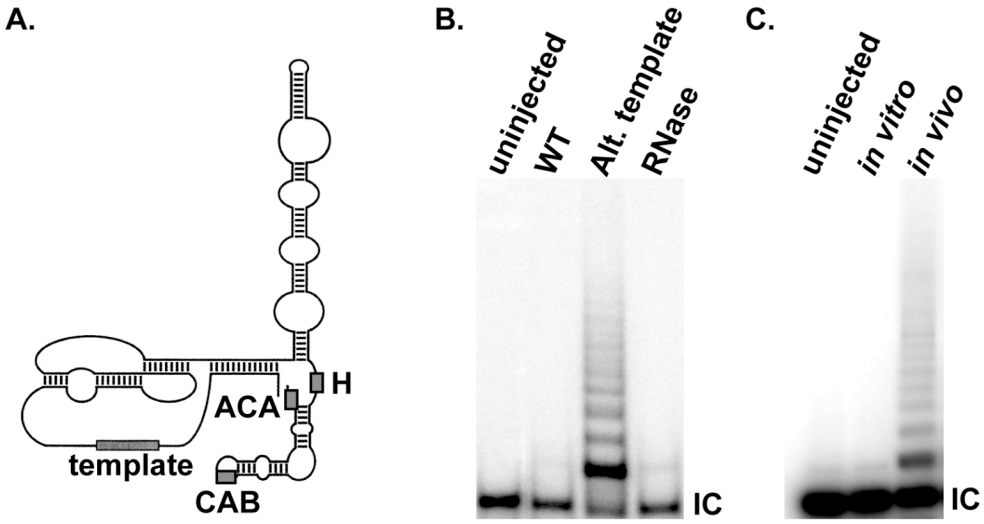

A.
H
ACA
template
CAB
B.
uninjected
WT
Alt. template
RNase
IC
C.
uninjected
in vitro
in vivo
IC

Figure A2.2. Injected xTR rapidly associates with nuclear structures before residing in the nucleoplasm in *Xenopus* oocytes. *(A) Framework of the experiments in this manuscript.* xTR (wild-type or a sequence variant) is injected into *Xenopus* oocyte nuclei. At specific time points following injection, nuclei are isolated and analyzed. Three tests, biochemical fractionation, nuclear spreads and TRAP assays, were performed to detect the distribution, subnuclear localization and activity of the injected RNA, respectively. *(B) Distribution of injected radiolabeled xTR in Xenopus oocytes.* ^{32}P-labeled xTR and control RNAs U3 and U1 were co-injected into oocyte nuclei. The nuclei were obtained and biochemical fractionation was perfomed at the indicated time points. The RNAs present in structural (pellet) and nucleoplasmic (supernatant) fractions are shown. U3 is a snoRNA that stably associates with nucleoli. U1 is a snRNA found primarily in the nucleoplasm. *(C) Graph of the distribution of xTR with relation to U3 and U1 control RNAs.* The results in (B) were quantitated using and the percent (%) of each indicated RNA found in the pellet is graphed relative to time (in hours) after injection. The results of three independent experiments are plotted; error bars indicate the standard deviation.

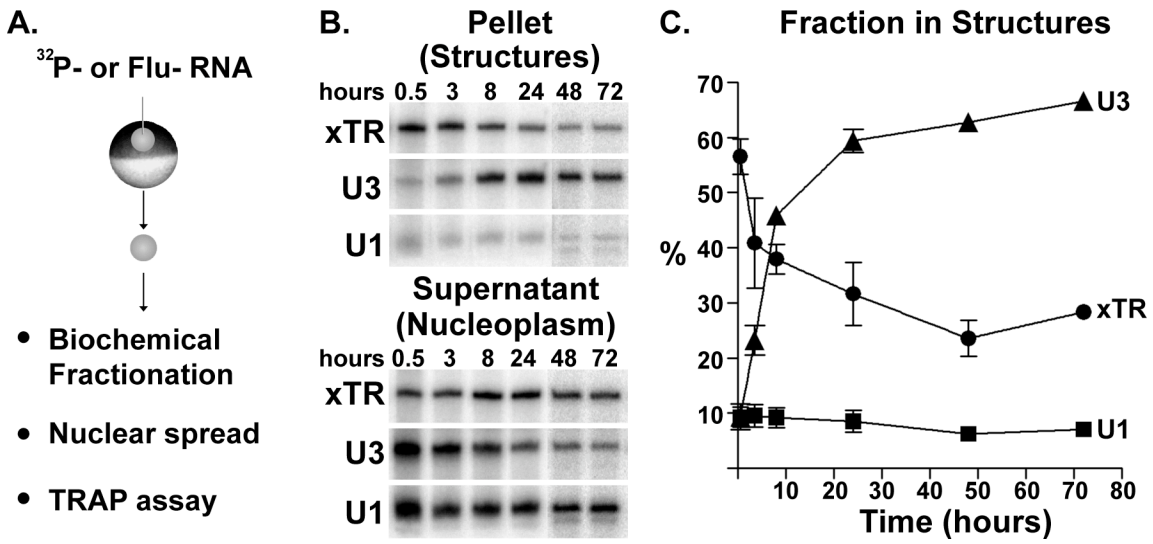

A.
32P- or Flu- RNA
• Biochemical Fractionation
• Nuclear spread
• TRAP assay
B.
Pellet (Structures)
hours 0.5 3 8 24 48 72
xTR
U3
U1
Supernatant (Nucleoplasm)
hours 0.5 3 8 24 48 72
xTR
U3
U1
C.
Fraction in Structures
%
70
60
50
40
30
20
10
U3
xTR
U1
10 20 30 40 50 60 70 80
Time (hours)

Figure A2.3. xTR rapidly localizes to Cajal bodies following injection into *Xenopus* oocytes. Fluorescein-labeled xTR (xTR panels) was injected (1 fmol/cell) and nuclear spreads were prepared at the indicated time points after injection. Cajal bodies were immunostained with coilin antibodies (coilin panels) and are indicated with arrowheads. Uninj, uninjected nucleus; DIC, differential interference contrast. The larger structures present in the DIC field are nucleoli. Bar: 10 μm

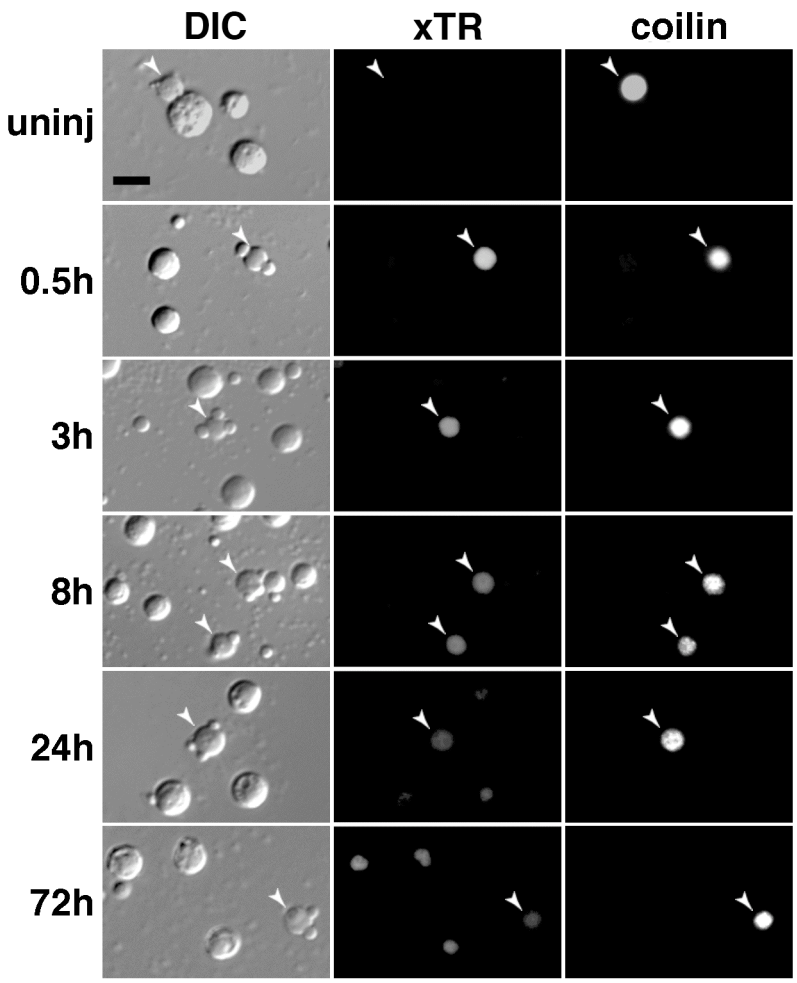

DIC
xTR
coilin
uninj
0.5h
3h
8h
24h
72h

Figure A2.4. Telomerase assembly happens quickly and the active enzyme is distributed mainly in the nucleoplasm. *(A) Injected xTR assembles rapidly with endogenous hTERT.* Altered template xTR was injected into oocyte nuclei (1 fmol/cell). At the indicated times (in hours) after injection, nuclei were collected and fractionated. The resulting supernatants and pellets were subjected to modified TRAP assay (specific for the injected xTR). Unfractionated nuclear extracts (total) were analyzed in tandem. -, negative control (no lysate); uninj, uninjected oocytes, IC, internal control for TRAP assay. *(B) Endogenous telomerase activity resides in the nucleoplasm.* Uninjected nuclei were fractionated, and telomerase activity in the pellet (P) and supernatant (S) were detected using the standard TRAP assay. Activity in total (not fractionated) extract (T) was analyzed as well. The distribution of xTR, U3, and U1 RNAs from the same fractionation was visualized by Northern analysis in the bottom panel.

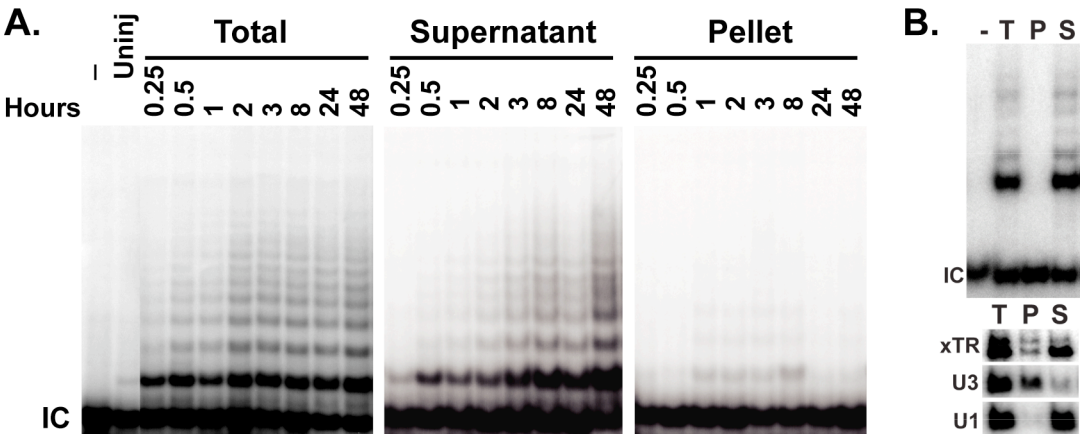

A.
Total
Supernatant
Pellet
–
Uninj
Hours
0.25
0.5
1
2
3
8
24
48
IC
B.
- T P S
IC
T P S
xTR
U3
U1

Figure A2.5. Nucleolar localization of xTR is not required for enzyme assembly. *(A) Box H and ACA mutant xTRs still assemble into an active enzyme in vivo.* Oocyte nuclei were harvested 1 and 3 hours after injection of ^{32}P-labeled altered template wildtype (WT) and mutant (H and ACA) xTRs. The nuclear extract was then subjected to the modified TRAP assay. IC, internal control for TRAP assay; -, no lysate control; uninj, uninjected nuclei. *(B) Box H and ACA mutant xTRs are less stable than wildtype xTR.* The RNAs present in the oocyte nuclei from (A) were isolated and analyzed by gel electrophoresis. U3 served as an injection and loading control. M, marker for amount of RNA injected. *(C) ACA mutant xTRs are assembled into active telomerase complexes more slowly than wildtype.* ACA mutant xTR (1fmol/cell) and a range of levels of wild type xTR (1, 0.2, 0.04 and 0.01 fmol/cell) were injected into Xenopus nuclei (all RNAs had altered template regions). The nuclei were isolated at 1 and 3 hours after injection and modified TRAP assays were performed on the nuclear lysates. At each time, RNAs were extracted and analyzed by gel electrophoresis (bottom panel). Arrows denote levels of wild type and ACA mutant RNA that give comparable activity at 1 and 3 hours. *(D) Box H/ACA mutant xTRs do not localize to nucleoli, but do associate with Cajal bodies.* Fluorescently labeled wildtype (WT panels) or mutant (H and ACA panels) xTR was injected into *Xenopus* oocytes (2 fmol/cell) and nuclear spreads were prepared 1 hr after injection (xTR panels). Cajal bodies were immunostained with coilin antibodies (coilin panels) and are indicated with arrowheads. DIC, differential interference contrast. The large spherical structures seen in the DIC panels correspond to nucleoli. Bar: 10μm

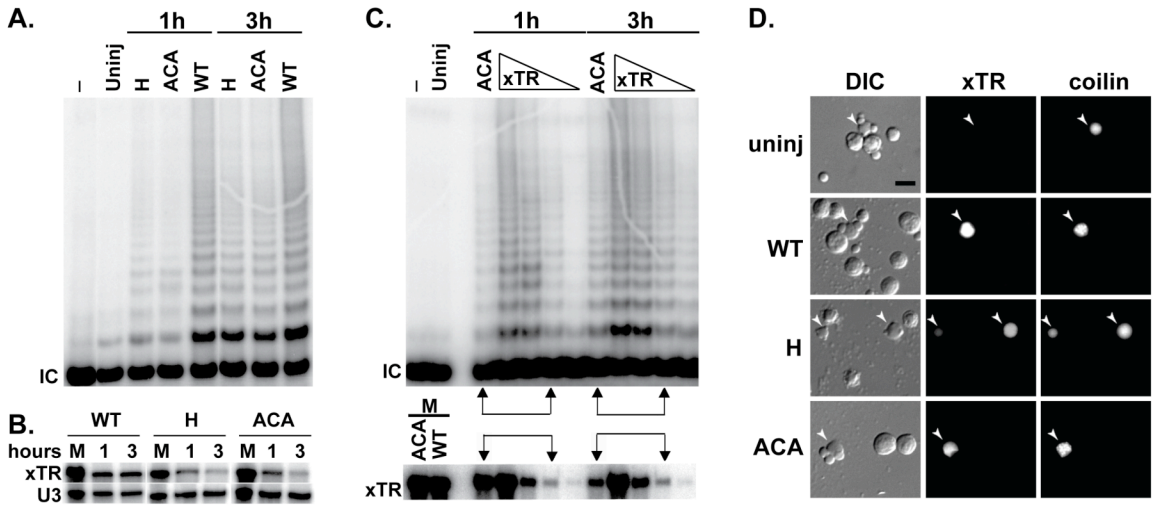
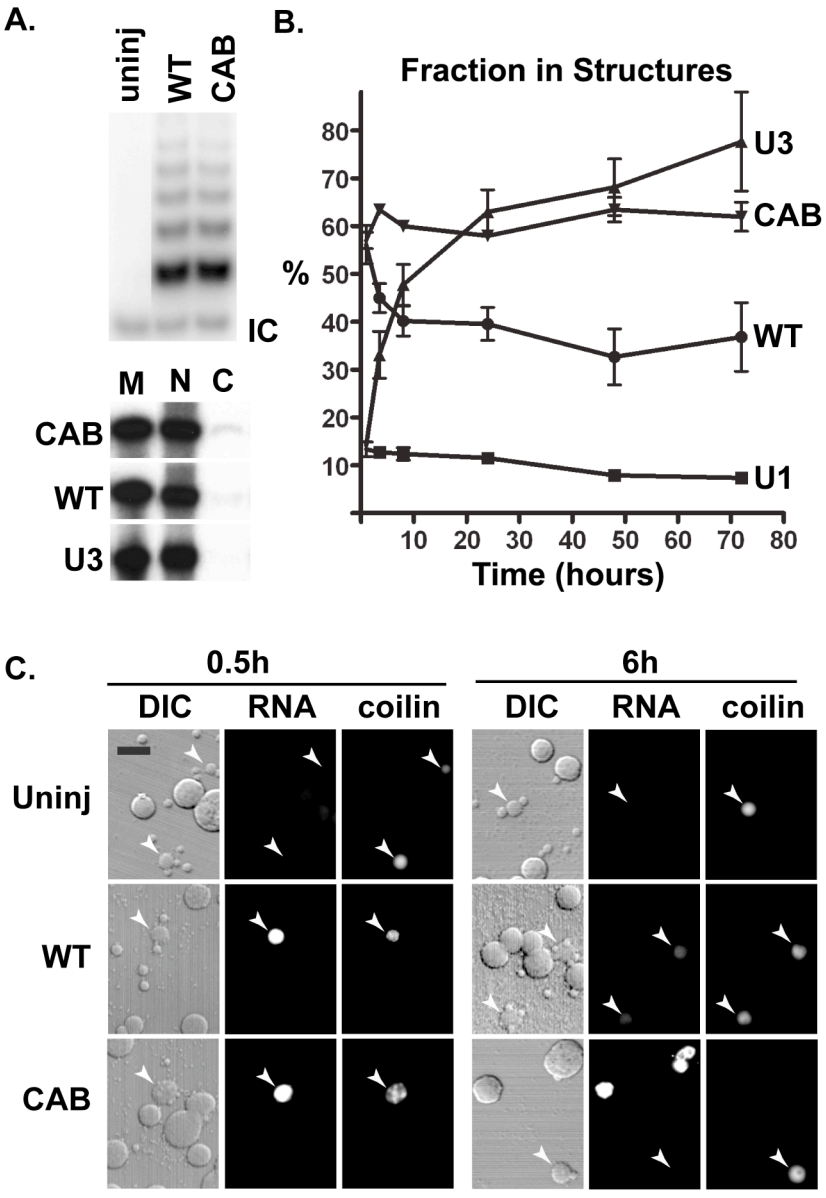

A.
1h
3h
–
Uninj
H
ACA
WT
H
ACA
WT
IC
B.
WT
H
ACA
hours M 1 3 M 1 3 M 1 3
xTR
U3
C.
1h
3h
–
Uninj
ACA
xTR
ACA
xTR
IC
M
ACA
WT
xTR
D.
DIC
xTR
coilin
uninj
WT
H
ACA

Figure A2.6. CAB mutant xTR assembles with endogenous xTERT and still associates with Cajal bodies in *Xenopus* oocytes. *(A) The CAB mutant is retained within the nucleus and can form active telomerase.* 1 fmol of wildtype (WT) or CAB mutant (CAB) xTR was injected into the nuclei of *Xenopus* oocytes (both RNAs had mutant templates as well). The activity (detected by modified TRAP assay, top panel) and stability (bottom panel) of each RNA was analyzed 1 hour after injection. U3 serves as an injection and loading control. Uninj, uninjected oocyte; IC, internal control; M, marker for amount of RNA injected; N, Nuclear; C, Cytoplasmic. *(B) The subnuclear distribution of CAB mutant xTR varies from that of wildtype.* Wildtype (WT) or CAB mutant (CAB) xTRs were co-injected with U3 and U1 control RNAs. The biochemical fractionation experiments were performed as described in Figure A2.2. The percent of each RNA present in the pelleted fraction (corresponding to subnuclear structures) is plotted versus time following injection (in hours). The graph represents the results of two independent experiments; error bars indicate standard deviation. *(C) CAB mutant xTR localizes to Cajal bodies prior to strongly associating with nucleoli.* 1 fmol of fluorescently labeled WT or CAB mutant xTR was injected (xTR panels). Nuclear spreads were prepared 0.5 and 6 hours after injection (as indicated). Cajal bodies were immunostained with coilin antibodies (coilin panels, denoted by arrowheads). Uninj, uninjected nuclei; DIC, differential interference contrast. The larger spherical structures present in the DIC panel are nucleoli. Bar: 10μm.


A.
uninj
WT
CAB
IC
M N C
CAB
WT
U3
B.
Fraction in Structures
%
80
70
60
50
40
30
20
10
U3
CAB
WT
U1
10 20 30 40 50 60 70 80
Time (hours)
C.
0.5h
6h
DIC
RNA
coilin
DIC
RNA
coilin
Uninj
WT
CAB