

Human telomerase RNA and box H/ACA scaRNAs share a common Cajal body–specific localization signal

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Telomerase is a ribonucleoprotein reverse transcriptase that uses its RNA component as a template for synthesis of telomeric DNA repeats at the ends of linear eukaryotic chromosomes. Here, fluorescence in situ hybridization demonstrates that in HeLa cancer cells, human telomerase RNA (hTR) accumulates in the nucleoplasmic Cajal bodies (CBs). Localization of transiently expressed hTR to CBs is supported by a short sequence motif (411-UGAG-414) that is located in the 3'-terminal box H/ACA

RNA-like domain of hTR and that is structurally and functionally indistinguishable from the CB-specific localization signal of box H/ACA small CB-specific RNAs. In synchronized HeLa cells, hTR shows the most efficient accumulation in CBs during S phase, when telomeres are most likely synthesized. CBs may function in post-transcriptional maturation (e.g., cap hypermethylation of hTR), but they may also play a role in the assembly and/or function of telomerase holoenzyme.

Introduction

The termini of eukaryotic chromosomes are capped with simple sequence repeats that form specific chromatin structures, called telomeres, which protect chromosomes from undesirable recombination, end-to-end fusion, and nucleolytic degradation (de Lange, 2002). However, due to the fact that DNA polymerases cannot fully replicate linear DNAs, the termini of telomeric DNAs are shortened with each round of genome replication. Eventually, erosion of telomeres below a critical length induces cell cycle arrest known as replicative senescence (McEachern et al., 2000).

Telomere attrition is counterbalanced by the telomerase reverse transcriptase that adds tandem DNA repeats onto the protruding 3' end of telomeric DNA (Collins and Mitchell, 2002). Telomerase is an RNP enzyme that contains at least two essential components; the telomerase RNA that provides the template sequence for telomere synthesis, and the telomerase reverse transcriptase. The human telomerase RNA (hTR) possesses two major structural domains (Chen et al., 2000; Fig. 1). The 5'-terminal part of hTR, which carries the template sequence, folds into a pseudoknot structure. The 3'-terminal region of hTR possesses all structural

hallmarks of box H/ACA small nucleolar RNAs (snoRNAs). It folds into two hairpin structures that are connected and followed by short single-stranded hinge and tail regions carrying the conserved H (AnAnnA) and ACA boxes, respectively (Mitchell et al., 1999; Chen et al., 2000). Moreover, RNP proteins associated with box H/ACA snoRNAs are also integral components of the human telomerase RNP (Filipowicz and Pogacic, 2002). The box H/ACA snoRNA-like domain of hTR is essential for both accumulation and in vivo function of telomerase (Fu and Collins, 2003). The H/ACA domain of hTR also contains telomerase-specific elements that are conserved at least in vertebrates (Chen et al., 2000). An internal stem-loop region in the 5' hairpin, called the CR4-CR5 domain (CR stands for conserved region), is required for telomerase activity (Mitchell and Collins, 2000; Bachand and Autexier, 2001; Chen et al., 2002). Another conserved region (CR7) in the terminal stem-loop of the 3' hairpin is essential for RNA accumulation (Fu and Collins, 2003).

Most box H/ACA snoRNAs function as guide RNAs in the site-specific pseudouridylation of rRNAs in the nucleolus (Kiss, 2001; Filipowicz and Pogacic, 2002). Recently, we have discovered a novel class of box H/ACA guide RNAs which, instead of accumulating in the nucleolus, specifically

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Abbreviations used in this paper: CAB box, Cajal body box; CB, Cajal body; hTR, human telomerase RNA; pol II, RNA polymerase II; scaRNA, small Cajal body–specific RNA; SMN, survival of motor neurons; snoRNA, small nucleolar RNA; TMG, 2,2,7-trimethyl guanosine.

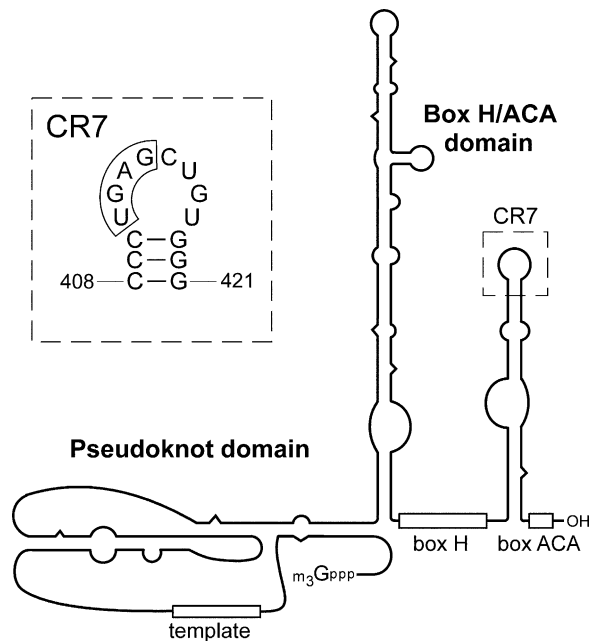


Figure 1. **Schematic structure of hTR.** The template region and the H and ACA boxes are indicated. The sequence of the conserved region 7 (CR7) is shown, and the putative CAB box motif is boxed.

localize to the nucleoplasmic Cajal bodies (CBs) and direct pseudouridylation of the RNA polymerase II (pol II)-specific spliceosomal snRNAs (Darzacq et al., 2002; Jády et al., 2003). These small Cajal body-specific RNAs (scaRNAs) carry two copies of a conserved sequence motif called the Cajal body box (CAB box) (Richard et al., 2003). CAB boxes are located in the terminal loops of the 5' and 3' hairpins of scaRNAs, and are composed of four nucleotides (consensus UGAG). The CAB boxes are cis-acting localization signals that direct the CB-specific accumulation of box H/ACA scaRNAs. In this paper, we demonstrate that in human HeLa cells, hTR specifically accumulates in CBs, and the 3'-terminal hairpin of the box H/ACA domain of hTR carries a CAB box motif that is responsible for the CB-specific accumulation of hTR.

Results and discussion

HeLa telomerase RNA accumulates in CBs

The majority of box H/ACA RNAs guide pseudouridylation of rRNAs in the nucleolus. Consistent with this, several lines of evidence suggest that hTR carrying a box H/ACA domain accumulates, at least partially, in the nucleolus (Mitchell et al., 1999; Narayanan et al., 1999; Lukowiak et al., 2001). However, discovery of human CB-specific box H/ACA scaRNAs (Darzacq et al., 2002) raised the possibility that hTR may also accumulate in CBs. Therefore, we investigated the subnuclear distribution of hTR in HeLa cells by FISH microscopy (Fig. 2). To detect hTR, we used a mixture of three fluorescent oligonucleotide probes complementary to different regions of hTR (Fig. 2). We found that HeLa hTR was highly enriched in a small number of dotlike nucleoplasmic foci (Fig. 2). Co-staining the same cells with antibodies directed against p80-coilin or the survival of mo-

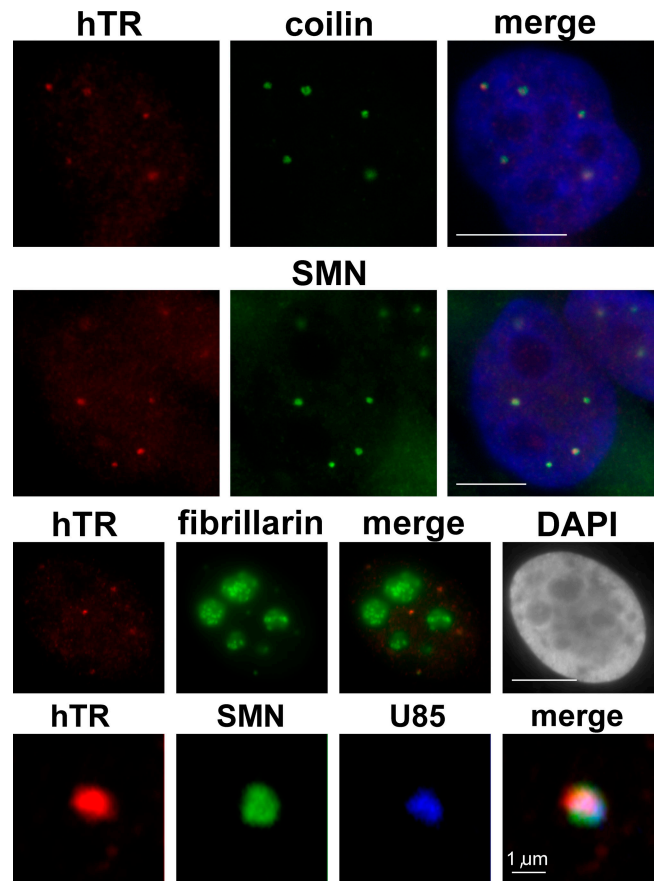


Figure 2. **In situ localization of endogenous hTR in HeLa cells.** HeLa cells were hybridized with a mixture of three fluorescent oligonucleotide probes complementary to hTR from G135 to A174, A346 to A382, and U416 to A449. The U85 scaRNA was detected with an oligonucleotide probe complementary to U85 from U13 to G53. CBs were stained with antibodies directed against p80-coilin or SMN. Nucleoli were visualized by transient expression of GFP-fibrillarin (Dundr et al., 2000). Nuclei were stained with DAPI. Unless indicated otherwise, bars represent 10 μ m.

tor neurons (SMN) protein, two marker proteins of the CB (Andrade et al., 1991; Liu and Dreyfuss, 1996), demonstrated that the nuclear organelles enriched in hTR were CBs. When the nucleoli of HeLa cells were labeled by transient expression of GFP-tagged fibrillarin, a nucleolar protein that also accumulates in CBs (Gall, 2000), hTR was rather excluded from the nucleolus. We also noticed that $\sim 20\%$ of the nucleoplasmic foci accumulating hTR did not perfectly overlap with bona fide CB structures that were defined by the presence of coilin, SMN, and the U85 canonical scaRNA (Darzacq et al., 2002). A magnified view of the distribution of hTR, SMN, and U85 is shown in the bottom panels of Fig. 2. This may indicate that hTR localizes to a specific subdomain of the CB, although this assumption needs further confirmation. Nevertheless, we concluded that hTR is a CB-specific RNA rather than a nucleolar RNA as believed before. This conclusion has been corroborated and further extended by a recent paper demonstrating that hTR accumulates in CBs not only in HeLa cells, but also in several other telomerase-positive tumor-derived cell lines (Zhu et al., 2004).

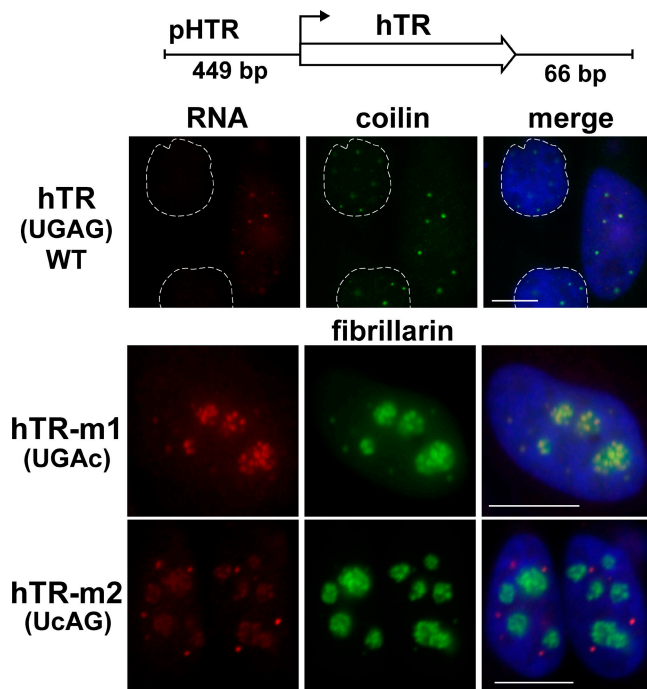


Figure 3. **CAB box-dependent localization of hTR to CBs.** Schematic structure of the hTR gene transfected into HeLa cells is shown. Sequences of the CAB box motifs of the wild-type (hTR) and mutant (hTR-m1 and hTR-m2) RNAs are shown. Altered nucleotides are indicated by lowercase letters. Cells were hybridized with a fluorescent probe complementary to hTR from A346 to A382. CBs were stained with an antibody against p80-coilin. Expression of GFP-fibrillarin visualized both nucleoli and CBs. Nuclei of nontransfected cells are circled. Bars, 10 μ m.

hTR carries a CAB box motif essential for localization to CBs

The CB-specific accumulation of box H/ACA scaRNAs is supported by a common localization signal, the CAB box (consensus UGAG), which is located in the terminal loops of their 5' and 3' hairpins (Richard et al., 2003). ScaRNAs carrying altered CAB boxes accumulate in the nucleolus like authentic box H/ACA snoRNAs. We noticed that the evolutionarily conserved CR7 sequence that forms the terminal stem-loop of the 3'-terminal hairpin of hTR carries a perfect CAB box motif (411-UGAG-414) (Fig. 1). To assess the functional importance of this putative CB localization signal of hTR, wild-type and mutant hTRs carrying altered nucleotides in their alleged CAB box motifs were transiently expressed in HeLa cells (Fig. 3). FISH performed with oligonucleotide probes specific for the 3'-terminal H/ACA domain (Fig. 3) or the 5'-terminal template domain (unpublished data) of hTR revealed that the overexpressed wild-type hTR localized to CBs. Due to the low concentration of hTR in HeLa cells, one oligonucleotide probe failed to detect endogenous hTR in the nucleus of nontransfected cells (Fig. 3, circled).

Previously, we have demonstrated that in the UGAG consensus of the CAB box, the last two nucleotides (A_3G_4) are crucial, whereas the first two nucleotides (U_1G_2) are less important for efficient localization of scaRNAs to CBs (Richard et al., 2003). Upon replacement of the G414 residue for

a C in the last position of the putative CAB box of hTR, the overexpressed mutant hTR-m1 RNA accumulated in large domains of the nucleus. Co-expression of GFP-fibrillarin, a marker protein of the nucleolus that is also present in CBs, demonstrated that hTR-m1 localized predominantly to nucleoli, although it was also detectable in CBs. Alteration of the G412 residue in the second position of the CAB box of hTR resulted in a mixed localization pattern: the mutant hTR-m2 RNA accumulated both in CBs and nucleoli.

These results demonstrate that the CB-specific localization of hTR is directed by the 411-UGAG-414 sequence motif that is structurally and functionally indistinguishable from the CAB box of canonical H/ACA scaRNAs. Most likely, localization of H/ACA scaRNAs and hTR to CBs is mediated by a common trans-acting protein factor that specifically binds to the CAB boxes of these RNAs. Mutations introduced into the CAB box of hTR inhibit binding of the putative CAB box protein, and therefore, the mutant telomerase RNAs are targeted into the nucleolus (Richard et al., 2003). However, despite the obvious mechanistic similarities, some differences may exist between the molecular mechanisms controlling the CB-specific accumulation of scaRNAs and hTR. The box H/ACA scaRNAs carry two CAB box motifs located in the terminal loops of their 5' and 3' hairpins (Richard et al., 2003). Efficient targeting of scaRNAs to CBs requires both CAB boxes. However, the terminal loop of the 5' hairpin of the H/ACA domain of hTR lacks an apparent CAB box motif. Therefore, it is unclear whether localization of hTR to CBs is supported exclusively by the 411-UGAG-414 CAB box element or whether hTR contains another (not yet identified) CAB box. CR7 encompassing the CAB box motif plays an essential role in hTR accumulation (Fu and Collins, 2003), indicating that this region of hTR has at least two functions. Although the 411-UGAG-414 CAB box directs the CB-specific accumulation of hTR, the rest of CR7 may direct the nucleolytic processing of hTR. As demonstrated by our mutational analysis, the two functions of CR7 can be uncoupled. Currently, we are dissecting the cis elements directing processing, accumulation, and localization of hTR.

Cell cycle-dependent accumulation of hTR in CBs

During in situ localization of HeLa hTR, we noticed that the amount of hTR accumulating in CBs highly varied when individual cells were compared in a population of unsynchronized cells (unpublished data). This suggested that the accumulation level of hTR in CBs may fluctuate during cell cycle. To test this assumption, HeLa cells were synchronized by double thymidine blocking, which arrests cells at G1/S transition (Galavazi et al., 1966). At 0 (G1/S), 2 (early S), 4 (late S), 7 (G2), and 12 (G1) h after being released from the second thymidine block, FACS[®] analysis (Crissman and Steinkamp, 1973) was performed to ensure that cells progressed in synchrony through the cell cycle, and accumulation of hTR in CBs bodies was monitored by FISH (Fig. 4 A). CBs were immunostained with anti-p80-coilin antibody. We found that accumulation of hTR in CBs persisted during cell division cycle. However, as compared with G1 or G2 cells, significantly more hTR was detected in CBs of early and late S cells. Quantification of the fluorescence

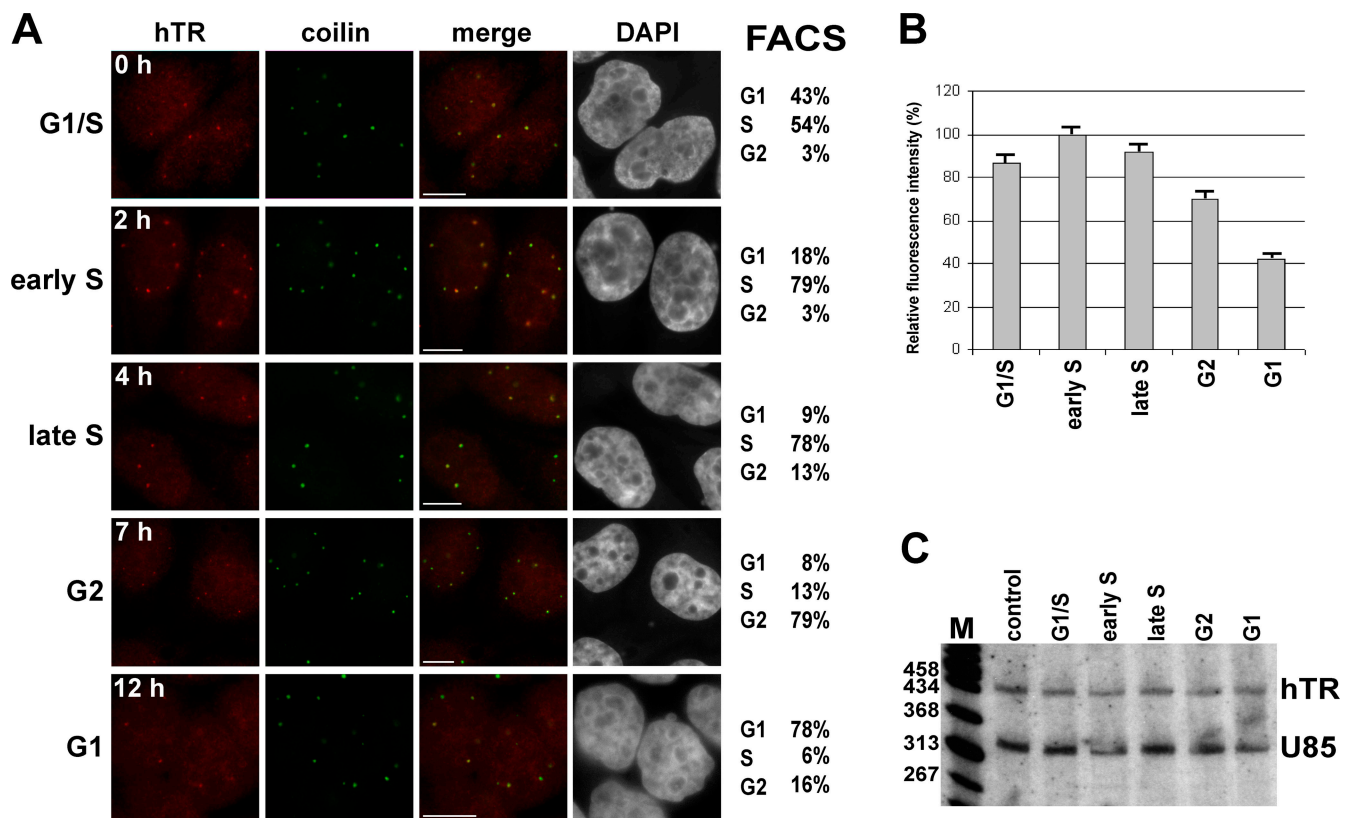


Figure 4. Cell cycle-dependent accumulation of hTR in CBs. (A) FISH. Human HeLa cells were synchronized by the double-thymidine blocking procedure. Progression of synchronized cells through cell cycle was monitored by FACS[®] analysis. The results of FACS[®] analysis calculated with CELLQuest software (Becton Dickinson) are indicated on the right. Each specimen was double stained with a mixture of hTR-specific fluorescent oligonucleotides and an anti-p80-coilin antibody. Bars, 10 μ m. (B) Quantification of hTR accumulation in CBs. To estimate the relative amount of hTR accumulating within CBs in different cell cycle phases, pictures of the endogenous hTR localization taken under identical conditions were analyzed with MetaMorph[®] software (Universal Imaging Corp.). For each CB, the difference between the maximum relative grayscale value of pixels representing the CB and the average relative grayscale value of pixels representing the surrounding nucleoplasm was determined. For each cell cycle phase, the obtained values for 100 randomly selected CBs were averaged. To define the relative value of fluorescence, the intensity of early S phase CBs was taken as 100%. Statistical analysis (Friedman's test and Tukey multiple comparisons test) confirmed that the differences between the measures obtained for S, G2, and G1 cells are significant at the 0.001 significance level. (C) Northern analysis. RNAs extracted from nonsynchronized (control) or synchronized HeLa cells in the early S, late S, G2, G1, and G1/S transition phases. 10- μ g RNA samples were analyzed by Northern blotting with a mixture of labeled oligonucleotides complementary to the U85 scaRNA and hTR. Lane M, size markers.

intensity of 100 randomly selected CBs in each cell cycle phase revealed that the amount of hTR sequestered into CBs increased by more than twofold during progression from G1 to early S phase (Fig. 4 B), indicating that hTR is recruited to CBs in a cell cycle-dependent manner. In contrast, accumulation of the U85 scaRNA in CBs showed no significant variation during cell cycle (unpublished data). Northern blot analysis demonstrated that the cellular level of hTR did not change during cell cycle (Fig. 4 C). This indicates that a fraction of hTR is likely present in the nucleoplasm at least during G1 and G2 phases and that, in contrast to canonical box H/ACA scaRNAs, hTR has a dynamic intranuclear localization pattern during cell cycle.

Does the CB function in the biogenesis of telomerase?

Accumulation of hTR in CBs strongly suggests that CBs may play a role in the biogenesis and/or function of telomerase RNP. This notion is supported by the fact that hTR shows an increased accumulation in CBs during S phase, when synthesis of telomeric DNA most likely occurs (Mar-

cand et al., 2000). Maturation of pol II-transcribed spliceosomal snRNAs and snoRNAs includes hypermethylation of their primary 7-monomethyl guanosine cap to 2,2,7-trimethyl guanosine (TMG). Although the primary cap of spliceosomal snRNAs is hypermethylated in the cytoplasm, synthesis of the 5'-terminal TMG cap of the U3 snoRNA, which is not transported to the cytoplasm, takes place in CBs (Verheggen et al., 2002). Because hTR is a pol II product (Feng et al., 1995), we tested whether it has a TMG cap. When HeLa cellular RNAs were immunoprecipitated with a monoclonal (H-20) and polyclonal (α -TMG) antibody directed against TMG (Fig. 5), both hTR and the U2 spliceosomal snRNA were precipitated, as demonstrated by RNase A/T1 mappings. In contrast, neither of the two antibodies recognized the U19 snoRNA that had a monophosphorylated 5' end. We concluded that hTR, like other pol II-specific snRNAs, possesses a TMG cap. However, in contrast to the pol II-specific spliceosomal snRNAs, hTR is not transported to the cytoplasm, it is retained in the nucleus (Lukowiak et al., 2001). Therefore, the primary cap of hTR

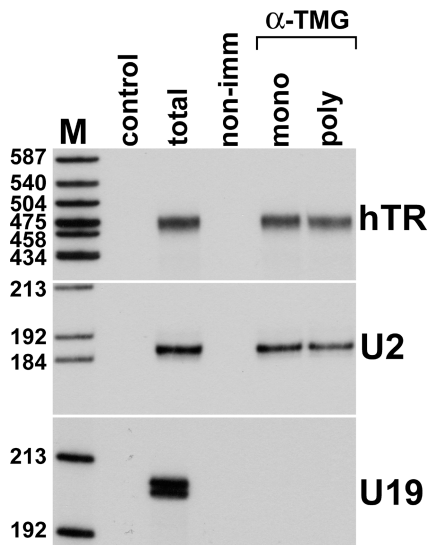


Figure 5. hTR carries a TMG cap. HeLa cellular RNAs immunoprecipitated with monoclonal (H-20) and polyclonal (α -TMG; provided by R. Lührmann, Max Planck Institute of Biophysical Chemistry, Göttingen, Germany) antibodies against TMG were mapped by RNase A/T1 protection using antisense RNA probes complementary to hTR, U2, and U19. Control mappings performed with *Escherichia coli* tRNA (control), HeLa total RNA (total), or RNA precipitated.

is hypermethylated most likely in CBs, as it has been shown for the U3 snoRNA. This conclusion is supported by the fact that the nuclear fraction of TGS1, the methyltransferase enzyme responsible for TMG synthesis, localizes to CBs (Verheggen et al., 2002). Because the CB has been established as the site of scaRNA-directed 2'-O-methylation and pseudouridylation of spliceosomal snRNAs (Darzacq et al., 2002; Jádý et al., 2003), hTR may also undergo internal modifications in CBs, although modified nucleotides have not yet been mapped for hTR.

The SMN protein plays an important role in the cytoplasmic assembly of spliceosomal snRNPs (Fischer et al., 1997; Meister et al., 2001; Pellizzoni et al., 2002). In addition to the cytoplasm, SMN is also present in the nucleus, where it is concentrated in CBs (Paushkin et al., 2002). Interestingly, SMN has been found to interact with the GAR1 box H/ACA RNP protein (Pellizzoni et al., 2001) and the human telomerase RNP (Bachand et al., 2002). This suggests that assembly of hTR with box H/ACA RNP proteins, and perhaps with human telomerase reverse transcriptase (hTERT), is assisted by SMN and occurs in CBs. Interestingly, accumulation of hTR in CBs has been found to require expression of hTERT (Zhu et al., 2004). On one hand, this finding lends further support to the idea that assembly of hTR and hTERT take place in CBs. On the other hand, it may explain why hTR accumulates in CBs only in telomerase-positive cancer cells, but not in primary cells that lack hTERT (Zhu et al., 2004). Finally, CBs may also function in the intranuclear trafficking of hTR. Consistent with this idea, in vivo imaging revealed that CBs are highly mobile organelles (Ogg and Lamond, 2002).

In conclusion, we have demonstrated that hTR specifically localizes to CBs of HeLa cancer cells by using an intranuclear targeting mechanism that is also responsible for the CB-spe-

cific accumulation of box H/ACA scaRNAs. The finding that hTR accumulates in CBs, besides implicating CBs in telomere synthesis, may open new perspectives in understanding of the complex regulation of human telomere synthesis.

Materials and methods

General procedures

Standard laboratory procedures were used for manipulating DNA and RNA. HeLa cells were grown in DME supplemented with 10% FCS (Invitrogen). Transfection was performed with FuGENE™ 6 (Roche) transfection reagent according to the manufacturer's instructions. Oligodeoxynucleotides used in this paper were as follows: (1) 5'-ATACTCGAGCTCGACGCATCCCACTGAG-3'; (2) 5'-ACAGGATCCACTGCCGCGAGGGGTGAC-3'; (3) 5'-GCGGCGCGATTCCCTGACCTGTGGGACGTGCAC-3'; (4) 5'-GCGGCGCGGATTCCCTGACCTGTGGGACGTGCAC-3'; (5) 5'-AT*CCGTTCCTCTT*CCTGCGGCCTGAAAGGCCTGAACCT*A-3'; (6) 5'-AT*TTGTTTGCTCT*AGAATGAACGGT*GGAAGCGCGCAGGCCT*A-3'; (7) 5'-AT*TGTGTGAGCCGAGTCTT*GGGTGCACGTCCACAT*A-3'; (8) 5'-CT*GGCTTAGCTAAACCAACT*GAATCACACAGCCTTGAT*A-3'; (9) 5'-GCGAACGGGCCAGCAGC-3'; (10) 5'-GCATGTGTGAGCCGAGTCTG-3'; and (11) 5'-GGCTTAGCCAAACCAACTG-3'. Aminoalyl-modified thymidines are marked by asterisks.

Plasmid construction

To construct pHTR, the hTR gene was PCR amplified using HeLa genomic DNA as a template and oligonucleotides 1 and 2 as upstream and downstream primers, respectively. The obtained PCR fragment was digested by XhoI and BamHI and inserted into pBlueScript® (Stratagene). pHTR-m1 and pHTR-m2 were generated by two consecutive PCR reactions. First, the 3' half of the hTR gene was amplified using oligonucleotide 2 as a common downstream primer and oligonucleotides 3 (m1) and 4 (m2) as mutagenic upstream primers. In the second amplification reaction, the obtained DNA fragments were used as 3' megaprimers together with the oligonucleotide 1 upstream primer. After digestion with XhoI and BamHI, the amplified fragments were inserted into pBlueScript®.

FISH, image acquisition, and processing

FISH with oligonucleotide probes has been described elsewhere (Darzacq et al., 2002). Sequence-specific oligonucleotide probes containing aminoalyl-T nucleotides were labeled with FluoroLink® Cy3 or Cy5 monofunctional reactive dye (Amersham Biosciences) and were used to detect transiently expressed hTR (oligonucleotide 5), the endogenous HeLa hTR (a mixture of oligonucleotides 5, 6, and 7), and U85 scaRNA (oligonucleotide 8). Human p80-coilin was detected with a polyclonal rabbit anti-coilin antibody (1:400 dilution; provided by A. Lamond, University of Dundee, Dundee, UK) followed by incubation with an anti-rabbit antibody-FITC conjugate (1:300 dilution; Sigma-Aldrich). SMN was detected by a monoclonal mouse anti-SMN antibody (1:500 dilution; BD Biosciences) in combination with an anti-mouse-FITC conjugate (1:100 dilution; Jackson ImmunoResearch Laboratories). Slides were mounted in mounting media containing 90% glycerol, 1× PBS, 0.1 μ g/ml DAPI, and 1 mg/ml *p*-phenylenediamine. Images were acquired at RT on a DMRA microscope (Leica) equipped for epifluorescence, with Leica PL APO lenses (100×/1.40–0.7) and with a CoolSNAP camera (Photometrics) controlled by MetaMorph® software (Universal Imaging Corp.). Images were pseudocolored with Adobe Photoshop®.

RNA analysis

RNA isolation, immunoprecipitation, and RNase A/T1 mapping were performed as described previously (Darzacq et al., 2002). For Northern analysis, 10 μ g total RNA was separated on a 4% denaturing polyacrylamide gel and electrophoretically transferred onto a Hybond-N nylon membrane (Amersham Biosciences). hTR was detected with a mixture of terminally ³²P-labeled oligonucleotides 9 and 10; accumulation of U85 scaRNA was monitored with oligonucleotide 11.

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