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Nucleolin Interacts with Telomerase*[§]

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Telomerase is a specialized reverse transcriptase composed of core RNA and protein subunits which plays essential roles in maintaining telomeres in actively dividing cells. Recent work indicates that telomerase shuttles between subcellular compartments during assembly and in response to specific stimuli. In particular, telomerase colocalizes with nucleoli in normal human fibroblasts. Here, we show that nucleolin, a major nucleolar phosphoprotein, interacts with telomerase and alters its subcellular localization. Nucleolin binds the human telomerase reverse transcriptase subunit (hTERT) through interactions with its RNA binding domain 4 and carboxyl-terminal RGG domain, and this binding also involves the telomerase RNA subunit *hTERC*. The protein-protein interaction between nucleolin and hTERT is critical for the nucleolar localization of hTERT. These findings indicate that interaction of hTERT and nucleolin participates in the dynamic intracellular localization of telomerase complex.

Telomeres are maintained by the ribonucleoprotein (RNP)¹ reverse transcriptase telomerase (1). The enzymatic core of human telomerase RNP is minimally composed of reverse transcriptase catalytic subunit (hTERT) and RNA component (*hTERC*) (2–5); however, additional factors also regulate telomerase function *in vivo* (6–8). Although expression of hTERT is a rate-limiting step for enzymatic activity of telomerase, additional regulatory steps, such as the assembly of *hTERC* and hTERT (9, 10) and intracellular trafficking of a telomerase

complex (11–13), play critical roles in the assembly and delivery of a biologically active telomerase complex to telomeres.

Much of the assembly of ribosomes and possibly other RNPs occurs in nucleoli (14, 15). Both hTERT and *hTERC* localize to nucleoli (11–13, 16–18). Nucleolin is a major nucleolar phosphoprotein, and nucleolin-specific antibodies have been used to identify nucleoli (19–21). Several studies implicate nucleolin as an RNA chaperone and/or shuttling protein for various host and viral components in nucleoli, the nucleoplasm, cytoplasm, and plasma membrane (19–21).

To understand telomerase RNP assembly, we wished to identify proteins that regulate hTERT nucleolar localization. Here we show that nucleolin interacts with hTERT in a manner dependent upon *hTERC* and that this interaction plays an important role in regulating the nucleolar localization of telomerase.

EXPERIMENTAL PROCEDURES

Plasmids—The bacterial and mammalian expression vectors for full sized nucleolin and nucleolin mutants as well as the mammalian expression vectors pNKZFLAG-hTERT (amino-terminal FLAG-tagged hTERT), pNCZFLAG-hTERT (carboxyl-terminal FLAG-tagged hTERT), and pNKZGST-hTERT (amino-terminal GST-fused hTERT) have been described previously (22–25). We generated an amino-terminal enhanced green fluorescence protein (EGFP)-hTERT fusion protein by replacing the sequence encoding the FLAG epitope in pNKZFLAG-hTERT with EGFP cDNA (pNKZEGFP-hTERT). EGFP cDNA was obtained by PCR using pLEGFP-C1 (BD Biosciences, Clontech) as a template. pGRN164 vector containing *hTERC* cDNA was linearized with FspI and used as a template for *hTERC* RNA preparation as described previously (5, 25).

Expression and Purification of Recombinant Proteins—GST-nucleolin fusion proteins were expressed and purified as described previously (26). Briefly, the transformed bacterial cells (BL21) were harvested by centrifugation and suspended in buffer A (phosphate-buffered saline (PBS) (–), 0.5% Triton X-100, 1 mM dithiothreitol). After centrifugation of the sonicated lysates, the supernatants were passed through DEAE-Sepharose, and the GST fusion proteins were recovered using glutathione-Sepharose 4B beads (Amersham Biosciences). The resin was washed, and the GST fusion proteins were then eluted with glutathione. The eluted solution was dialyzed against buffer B (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol) for 12 h. Amino-terminally FLAG-tagged, recombinant hTERT was produced using baculovirus expression system in High5 insect cells as described previously (5).

Preparation of Cell Extracts, Immunoprecipitation, and Immunoblotting—Cells were harvested, washed with PBS (–), and sonicated in a lysis buffer (50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 10 mM aprotinin, 1 mM dithiothreitol). Lysates derived from 5×10^6 cells were diluted 10-fold in the lysis buffer containing 1% bovine serum albumin and precleared by incubation with GammaBind G-Sepharose (Amersham Biosciences) at 4 °C for 1 h. Precleared lysates were incubated with 10 μ l of GammaBind G resin with prebound α -FLAG M2 (Sigma) or α -GST (Z-5, Santa Cruz) antibodies at 4 °C for 4 h. Resins were preblocked in lysis buffer containing 1% bovine serum albumin. After an extensive washing (50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 1 mM

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains Figs. S1–S3 and additional experimental procedures.

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¹ The abbreviations used are: RNP, ribonucleoprotein; ALT, alternative lengthening of telomere; EGFP, enhanced green fluorescence protein; GST, glutathione S-transferase; *hTERC*, human telomerase RNA subunit; hTERT, human telomerase reverse transcriptase subunit; PBS, phosphate-buffered saline; RBD, RNA binding domain; RdRP, RNA-dependent RNA polymerase; RGG, RGG rich domain of nucleolin; TRAP, telomere repeat amplification protocol.

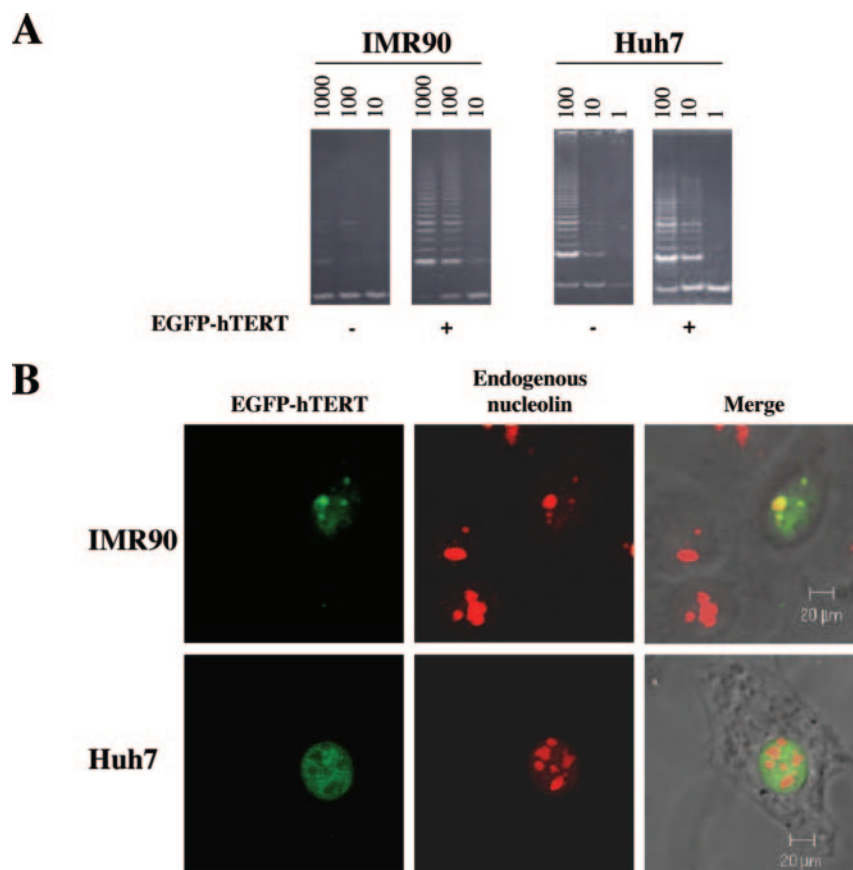


FIG. 1. A, telomerase activity in IMR90 and Huh7 cells expressing EGFP-hTERT. Telomerase activity was assessed by the TRAP assay. The number of cell equivalents loaded is indicated at the top of each lane. B, subcellular localization of EGFP-hTERT and nucleolin. IMR90 and Huh7 cells were seeded on glass slides and were transfected with EGFP-hTERT. Green, EGFP-hTERT; red, nucleolin; and yellow, merge).

EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 10 mM aprotinin, 1 mM dithiothreitol), the bound proteins were fractionated by SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to immunoblot analysis with monoclonal antibodies specific for GST (B-14, Santa Cruz) or the FLAG epitope (M2). For RNase treatment, the same lysates were divided into two tubes and incubated at room temperature for 15 min in the presence or absence of 0.1 $\mu\text{g}/\mu\text{l}$ RNase A followed by the addition of affinity resins. For *in vitro* GST pull-down experiments, 260 ng of recombinant GST-nucleolin fusion protein was mixed with 220 ng of recombinant FLAG-hTERT in the presence or absence of 250 ng of *in vitro* transcribed *hTERC* in a binding buffer containing 1% bovine serum albumin and 250 ng/ml yeast total RNA. The bound proteins were fractionated by SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to immunoblot analysis with α -GST (B-14) or α -FLAG M2 antibodies.

Cell Culture and Transient Transfection—IMR90 (normal human lung fibroblasts), Huh7, HLE (hepatoma cell lines), VA13 (SV40-transformed lung fibroblast, subline 2RA) and VA13+*hTERC* (VA13 cells with stable expression of *hTERC*) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (CELLect R GOLD) and 20 $\mu\text{g}/\text{ml}$ antibiotics (ampicillin and kanamycin; Meigs Co., Ltd.) and maintained in an incubator with 5% CO_2 at 37 °C. Cells were transfected using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's recommendations.

Immunofluorescence and Confocal Microscopy—Cells grown on glass slides were washed once in PBS (–) and fixed with 4% paraformaldehyde in PBS (–) for 20 min. Fixed cells were permeabilized by 0.5% Triton X-100 at room temperature for 10 min and blocked with 1% bovine serum albumin in PBS (–) for 1 h. Cells were then incubated with primary antibody in a humidifying chamber for 1 h, washed three times with PBS (–), and incubated with secondary antibody for 1 h. Subsequently, the cells were washed five times in PBS (–) and mounted using Vectashield mounting medium. Immunofluorescent images were acquired using a Confocal Laser Scanning Microscope (LSM510; Carl Zeiss Co., Ltd). To visualize endogenous nucleolin or GST fusion proteins, cells were stained with a rabbit polyclonal α -nucleolin antibody (1:500 (24)) or a rabbit polyclonal α -GST antibody (1:300, Z-5), respectively and visualized with a rhodamine-conjugated goat α -rabbit IgG (1:100, Pierce).

Telomerase Activity Assays and Immunoprecipitation-Telomerase Repeat Amplification Protocol (TRAP)—Total lysates of cells were subjected to TRAP assay using a TRAPEZE kit (Intergen) according to the manufacturer's method. For immunoprecipitation-TRAP, lysates from $\sim 2 \times 10^5$ cells/test tube were subjected to immunoprecipitation with a mouse monoclonal antibody specific for hTERT (2C4, subtype IgM (27)), with a rabbit polyclonal antibody specific for the amino terminus of nucleolin, or with an α -FLAG M2 antibody. As a control, lysates were immunoprecipitated with an isotype-matched, mouse monoclonal antibody specific for α -actinin (Sigma, clone BM-75.2, subtype IgM) or rabbit preimmune serum. The IgG subtype antibodies were bound directly to 10 μl of GammaBind G-Sepharose. The IgM subtype antibodies were bound to same amount of resin using rabbit polyclonal anti-mouse IgM antibody (Pierce). Telomerase activity was detected using 1 μl of post-immunoprecipitation resin. For *in vitro* reconstitution assays, for one test tube 100 ng of purified FLAG-hTERT, 200 ng *in vitro* transcribed *hTERC*, and increasing amounts of purified nucleolin-1234R were mixed in 20 μl of reconstitution buffer (5). After incubation at 33 °C for 10 min, samples were subjected to telomerase reaction using Telomerase kit (Toyobo).

RESULTS

Subcellular Localization of hTERT—To establish the subcellular localization of hTERT, we transiently expressed an EGFP-hTERT fusion protein in normal human lung fibroblasts (IMR90) and in a hepatoma-derived cancer cell line (Huh7). Ectopic expression of EGFP-hTERT conferred telomerase activity in IMR90 cells as well as a slight increase of telomerase activity in Huh7 cells (Fig. 1A), indicating that EGFP-hTERT formed an active telomerase complex. IMR90 cells expressing EGFP-hTERT showed a pan-nuclear localization of EGFP-hTERT with enriched nucleolar localization as assessed by costaining with an anti-nucleolin-specific antibody. In contrast, Huh7 cells expressing EGFP-hTERT exhibited a diffuse nucleoplasmic distribution of EGFP-hTERT with exclusion of EGFP-hTERT from the nucleoli (Fig. 1B), consistent with a prior report (11). We note that although the percentage of EGFP-

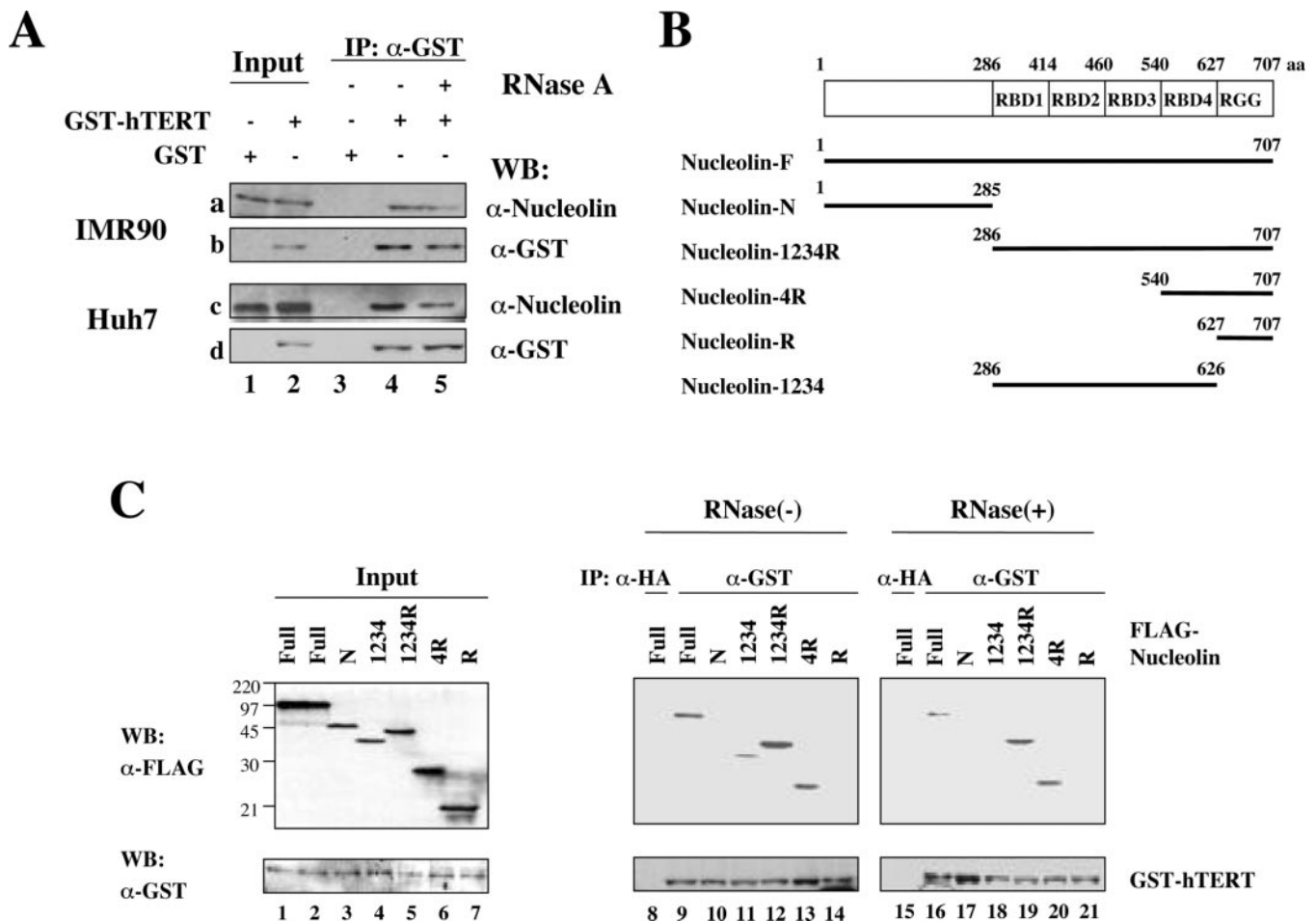


FIG. 2. A, hTERT interacts with endogenous nucleolin. Immune complexes formed after immunoprecipitation (IP) from total lysates from IMR90 and Huh7 cells transiently expressing GST alone (lane 3) or GST-hTERT with an α-GST antibody (lanes 4 and 5) were immunoblotted (WB) with α-nucleolin or with α-GST antibodies as indicated. Cell lysates were treated with RNase A before immunoprecipitation (lane 5). GST alone was used as a negative control (lanes 1 and 3). Immunoblotting by α-GST antibodies in b and d corresponds to GST-hTERT. Immunoblotting of total lysates is equal to 5% of the input shown in lanes 1 and 2. B, schematic presentation of full-sized nucleolin and truncation mutants. RBD1–4, RNA binding domains 1–4; RGG, RNA binding domain of nucleolin rich in RGG sequence. C, plasmids expressing GST-hTERT and FLAG-tagged nucleolin proteins were transiently cotransfected into Huh7 cells, and cell lysates were treated with RNase A (lanes 15–21) or mock treated (lanes 8–14). Treated lysates were immunoprecipitated by α-GST antibody and immunoblotted with α-FLAG or α-GST antibodies as indicated. α-HA antibody was used as a negative control. Immunoblotting of total lysates is equal to 5% of the input shown in lanes 1–7.

hTERT-expressing cells in the transfected population of IMR90 cells was lower than those in Huh7 cells (20 and 60% of the cell population, respectively), the distinct localization pattern of EGFP-hTERT between these cell lines was reproducible. It may be related to a lower expression level of *hTERT* in normal cells than that in transformed cells (data not shown) or reflect deregulation of shuttling process of hTERT between the nucleolus and nucleoplasm in transformed cells (see “Discussion”).

Interactions between hTERT and Nucleolin—The colocalization of EGFP-hTERT with nucleolin in nucleoli of normal cells, but not in cancer cells, suggested that nucleolin and hTERT physically interact. To examine this possibility, N-terminally GST-fused hTERT was transiently expressed in IMR90 or Huh7 cells. When we used an α-GST antibody to immunoprecipitate epitope-tagged hTERT from IMR90 cells, we found endogenous nucleolin in these immune complexes (Fig. 2A, a, lane 4). Despite the preferential nucleoplasmic localization of hTERT, endogenous nucleolin was also detected in α-GST-immunoprecipitates derived from Huh7 cells expressing GST-hTERT (Fig. 2A, c, lane 4). Endogenous nucleolin was similarly recovered in α-FLAG immunoprecipitates from IMR90 cells and Huh7 cells expressing carboxyl-terminally FLAG-tagged hTERT (data not shown). A possibility that interaction of hTERT and nucleolin occurs not in cells but in lysate after cell

disruption is unlikely because excess recombinant competitor proteins in lysates could not interfere with the interaction of the two protein (Supplemental data, Fig. S1). These observations indicate that the interaction of hTERT and nucleolin occurs in both normal and cancer cells, implying that the interaction of nucleolin and hTERT occurs both in the nucleolus and in the nucleoplasm.

Because the assembly of hTERT with *hTERT* may occur in the nucleolus similar to other RNPs (11–15), we speculated that the binding of hTERT with nucleolin required the presence of *hTERT*. When we treated total cell lysates with RNase A, we found that this treatment reduced but did not eliminate the amount of nucleolin recovered in the immunoprecipitates in both normal and cancer cells (Fig. 2A, lane 5). These findings suggest that one or more RNA component(s) contributes to the interaction between nucleolin and hTERT.

To confirm the specific interaction between nucleolin and hTERT, we mapped the regions of nucleolin required for its interaction with hTERT. For these experiments, we transiently coexpressed FLAG epitope-tagged, full-length nucleolin or a series of FLAG-nucleolin truncation mutants (Fig. 2B) with a GST-hTERT fusion protein into Huh7 cells. Using an α-GST antibody, we found that full-length nucleolin and two nucleolin truncation mutants, nucleolin-1234R and nucleolin-4R, bound

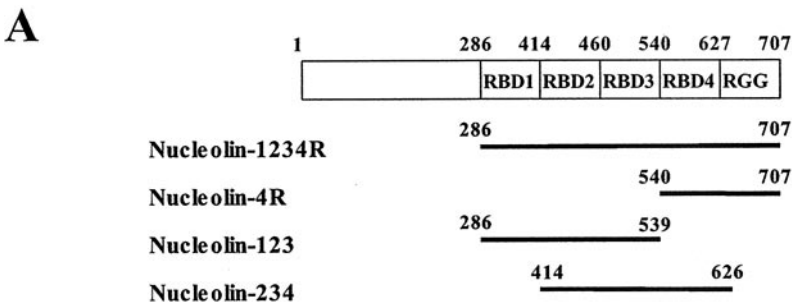
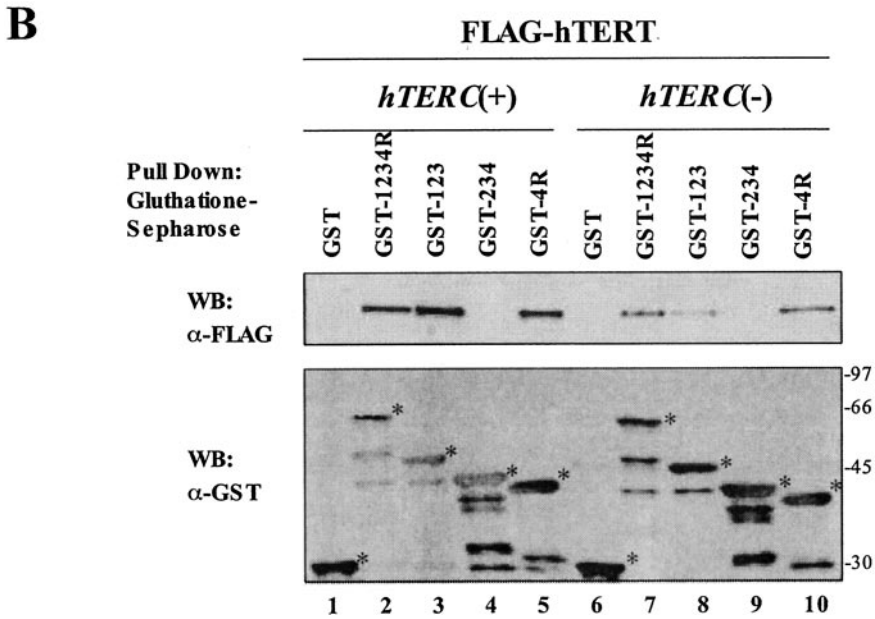


FIG. 3. Interaction of nucleolin and hTERT *in vitro*. A, schematic presentation of nucleolin truncation mutants used for the *in vitro* binding assay. The full sized nucleolin as well as the N-terminal of nucleolin were not examined because these constructs could not be expressed in *E. coli*. B, GST-nucleolin fusion proteins were expressed and purified from *E. coli* and then were mixed with partially purified recombinant FLAG-hTERT protein or with *in vitro* reconstituted telomerase. The mixtures were subjected to pull-down assay with glutathione-Sepharose 4B resin and blotted (WB) with α -FLAG or α -GST antibodies as indicated. Specific bands for GST and GST-nucleolin fusion proteins are indicated by asterisks.



		hTERT binding			
		hTERC (+)		hTERC (-)	
		in vivo	in vitro	in vivo	in vitro
1	286 414 460 540 627 707 aa				
	RBD1RBD2RBD3RBD4RGG				
Nucleolin-F	1707	++	nd	+	nd
Nucleolin-N	1285	-	nd	-	nd
Nucleolin-1234R	286707	++	++	+	+
Nucleolin-4R	540707	++	++	+	+
Nucleolin-R	627707	-	nd	-	nd
Nucleolin-1234	286626	++	nd	-	nd
Nucleolin-123	286539	nd	++	nd	-
Nucleolin-234	414626	nd	-	nd	-

FIG. 4. Structure of human nucleolin and its truncation forms with summary of their binding ability to hTERT. The binding ability of each mutant to hTERT in the presence or absence of hTERC is summarized on the right (+, binding positive; ++, stronger binding; nd, not determined).

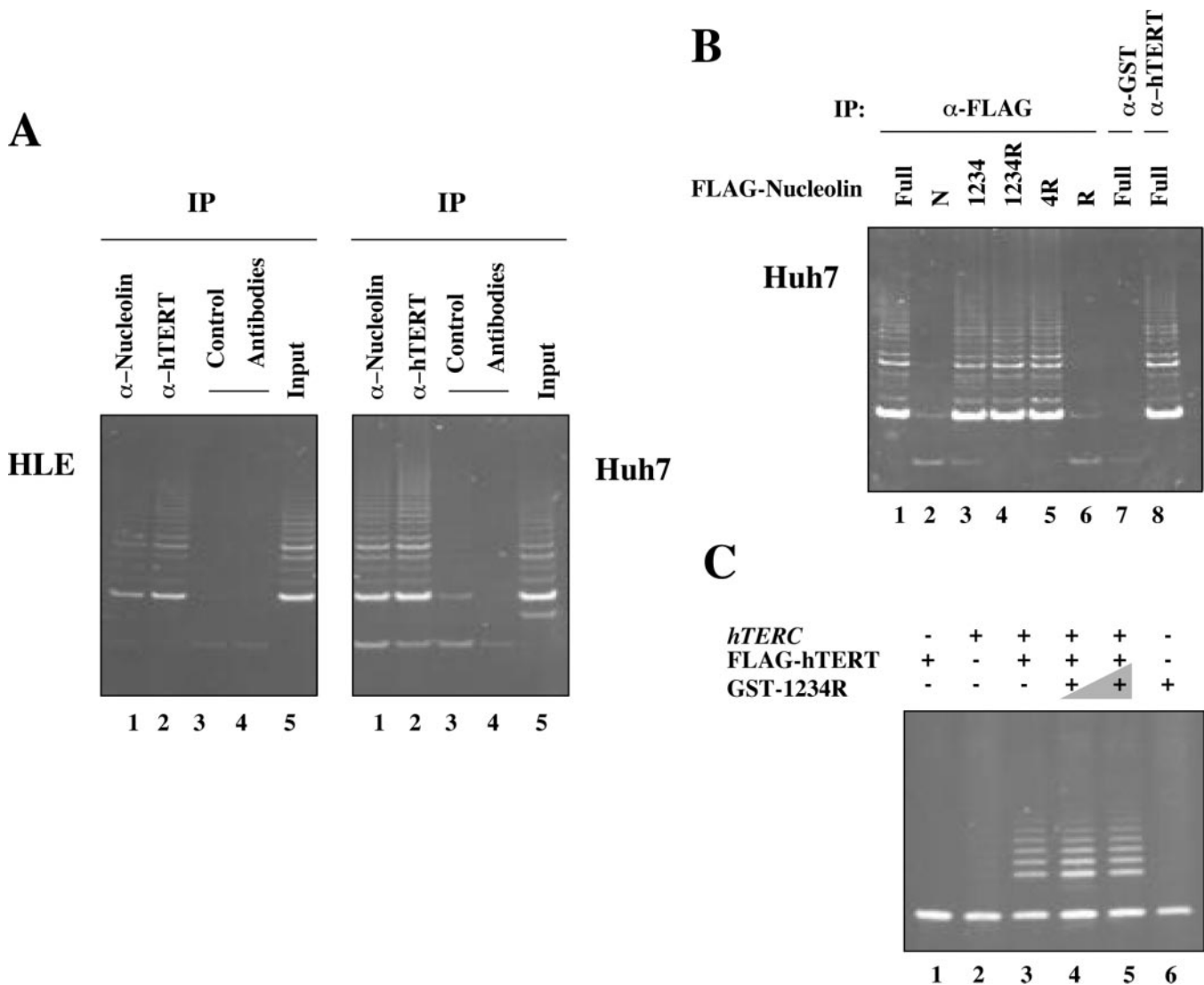


FIG. 5. Telomerase activity in nucleolin immune complexes. A, Huh7 and HLE lysates derived from 1×10^3 cells were subjected to TRAP assay (lane 5). The same lysates from $\sim 2 \times 10^5$ cells/test tube were immunoprecipitated (IP) with an antibody specific for hTERT (lane 2) or with an α -nucleolin antibody (lane 1). As a control, lysates were immunoprecipitated with an antibody specific for α -actinin (lane 3) or rabbit preimmune serum (lane 4). Telomerase activity was determined using 1 μ l of post-immunoprecipitation resin. B, Huh7 cells were transiently transfected with plasmids expressing FLAG-nucleolin proteins. Cell lysates were subjected to immunoprecipitation with an α -FLAG antibody (lanes 1–6) or antibody specific for hTERT (lane 8). Immunoprecipitation with α -GST antibody was used as a negative control (lane 7). TRAP assay was done as described in A. C, effect of nucleolin-1234R on *in vitro* reconstituted telomerase activity. Partially purified, recombinant FLAG-hTERT expressed in insect cells was reconstituted with *in vitro* transcribed *hTERC* in the absence (lane 3) or the presence (lanes 4 and 5) of an increasing amount (200–400 ng, respectively) of *E. coli* expressed and purified nucleolin-1234R. Telomerase activity was assessed using a Telochaser kit.

GST-hTERT. In contrast, truncation mutants containing the amino terminus of nucleolin and RGG domain alone failed to bind GST-hTERT (Fig. 2C). Interestingly, the binding of nucleolin-1234 to hTERT was abrogated by RNase A treatment (Fig. 2C, lanes 11 and 18). These observations indicate that nucleolin harbors two regions responsible for hTERT binding. The region within 4R binds only through protein-protein interactions, whereas the region within 1234 requires the involvement of RNA. RNase A treatment reduced the amount of hTERT recovered in the immunocomplexes containing full sized nucleolin or nucleolin-1234R. This finding is consistent with the view that both protein-protein and protein-RNA interactions contribute additively to the binding of hTERT and nucleolin.

Direct Binding of hTERT and Nucleolin *In Vitro*—To determine whether the binding of nucleolin and hTERT was direct, we prepared partially purified bacterial GST-nucleolin proteins, partially purified insect cell-expressed FLAG-hTERT alone, and purified *hTERC* and ascertained whether these

partially purified proteins could bind hTERT in the presence and absence of *hTERC* (Fig. 3). Nucleolin-1234R, -123, and -4R but not nucleolin-234 or GST alone bound reconstituted hTERT (Fig. 3B, lanes 1–5). The direct interaction of hTERT and nucleolin was clearly detected with nucleolin-1234R and nucleolin-4R (Fig. 3B, lanes 7 and 10). The minimum region for the direct hTERT binding was 4R, consistent with the cellular analysis (Fig. 2). Nucleolin-123 also bound hTERT *in vitro*, although this interaction was much weaker than that with the reconstituted telomerase (Fig. 3B, lanes 8 and 3). However, RNase A treatment of total cell lysates eliminated the interaction between nucleolin-1234 and hTERT (Fig. 2C, lane 18). This discrepancy may be because of the use of higher amounts of the purified proteins *in vitro* than those in the mammalian cell lysates. Alternatively nucleolin-123 may retain weak hTERT binding ability. The augmentation effect of *hTERC* on the direct binding between hTERT and nucleolin *in vitro* (Fig. 3) implies that *hTERC* is a major RNA that contributes to the interaction between nucleolin and telomerase. The strong stim-

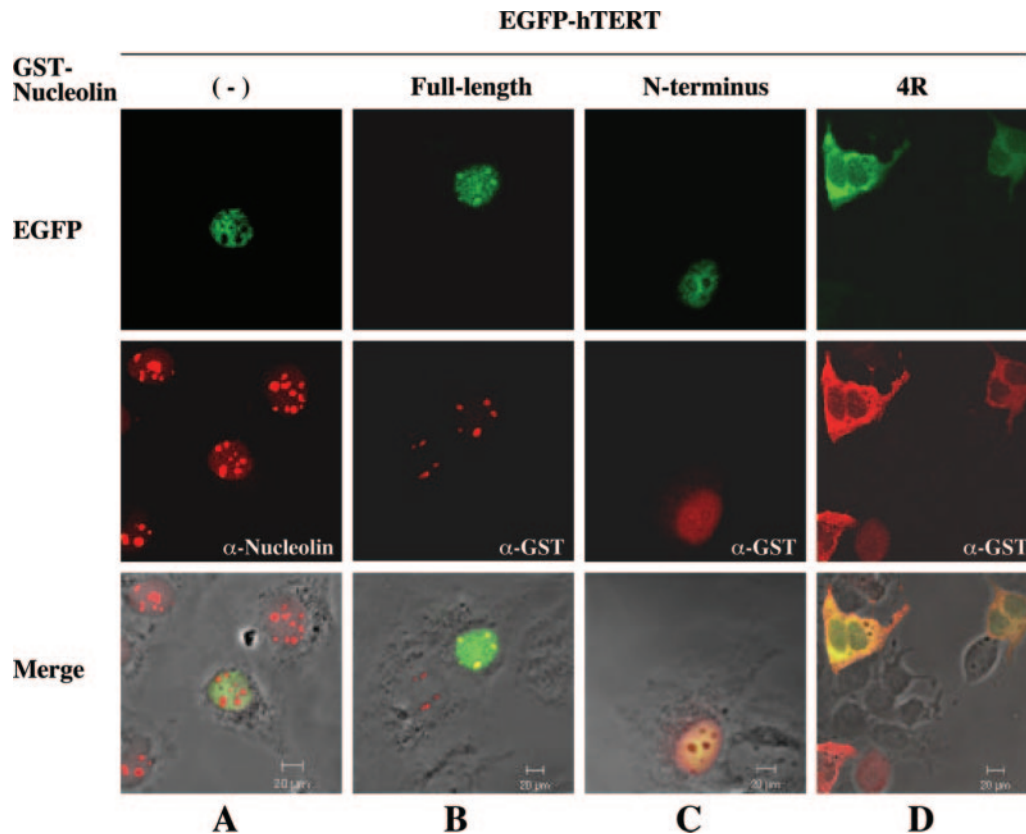


FIG. 6. **Nucleolin alters the subcellular localization of hTERT.** EGFP-hTERT was expressed alone (A) or together with GST-nucleolin fusion proteins in Huh7 cells. Full-length (B), nucleolin N terminus (C), and nucleolin-4R (D) mutants were used. After transfection cells were fixed and stained with an α -nucleolin (A) or α -GST antibodies (B–D). Green, EGFP-hTERT; red, nucleolin; and yellow, merge.

ulating effect of *hTERT* on the binding between hTERT and nucleolin-123, but not nucleolin-234, may indicate that the RBD1 is the *hTERT* binding region. Taken together, these results indicate that the specific interaction of telomerase and nucleolin requires RBD4 and the RGG domains of nucleolin and that *hTERT* is partly involved in this interaction. However, RBD1 may also contribute to the binding of telomerase with nucleolin through interactions with *hTERT* (Fig. 4).

Telomerase Activity in Nucleolin Immune Complexes—To determine whether endogenous nucleolin and telomerase interact, we performed immunoprecipitation experiments with an α -nucleolin antibody and found that we could detect telomerase activity in these immune complexes (Fig. 5, A and B). Moreover, when we performed similar experiments using cells expressing the nucleolin-N and nucleolin-R mutants, which are unable to bind hTERT, we failed to recover telomerase activity (Fig. 5B), confirming that recovery of telomerase activity depends upon the interaction between telomerase and nucleolin. These findings support the notion that endogenous nucleolin, at least in part, interacts with the telomerase complex. Moreover, these observations indicate that the interaction of nucleolin with hTERT does not inhibit telomerase activity.

To address whether nucleolin modulates telomerase activity, we developed an *in vitro* reconstituted telomerase activity assay. The addition of purified recombinant human nucleolin-1234R failed to augment or inhibit *in vitro* reconstituted telomerase activity even when it was added in 8-fold molar excess to hTERT (Fig. 5C). These results suggest that nucleolin does not modulate telomerase enzymatic activity.

Nucleolin Affects Subcellular Localization of hTERT—Because nucleolin and hTERT interact and colocalize, we addressed whether nucleolin affects the subcellular localization of hTERT. Full-length and truncated forms of nucleolin were

expressed as GST fusion proteins in Huh7 cells expressing EGFP-hTERT (Fig. 6). Although we observed diffuse GFP signals in the nucleoplasm, coexpression of full sized nucleolin resulted in enriched localization of EGFP-hTERT in nucleoli (Fig. 6B). In contrast, coexpression of nucleolin-4R, the fragment of nucleolin which binds hTERT, with EGFP-hTERT showed cytoplasmic distribution (Fig. 6D). However, coexpression of the nucleolin-N mutant with EGFP-hTERT failed to affect the localization of EGFP-hTERT (Fig. 6C). Taken together, these observations demonstrate that nucleolin affects the subcellular localization of hTERT.

To confirm these observations, we examined subcellular localization of EGFP-hTERT using VA13 cells, which maintain their telomeres through an ALT mechanism rather than through the expression of telomerase. This cell line lacks both *hTERT* and hTERT (30, 31). Interestingly, expression of EGFP-hTERT in VA13 cells resulted in nucleolar localization of EGFP-hTERT in the absence of *hTERT*. The further introduction of *hTERT* shifted this localization of EGFP-hTERT to the nucleoplasm (Fig. 7A). EGFP-hTERT expression resulted in readily detectable telomerase activity in VA13+hTERT cells, although VA13 cells failed to show any telomerase activity upon expressing of EGFP-hTERT (Fig. 7B). FLAG-hTERT immune complexes derived from both VA13 and in VA13+hTERT contained endogenous nucleolin (Fig. 7C). Taken together, the results show that the interaction of nucleolin with hTERT and *hTERT* regulates the subcellular localization of telomerase.

DISCUSSION

The regulation of subcellular localization of hTERT, the catalytic subunit of the telomerase complex, may regulate the biological functions of telomerase by controlling access of the

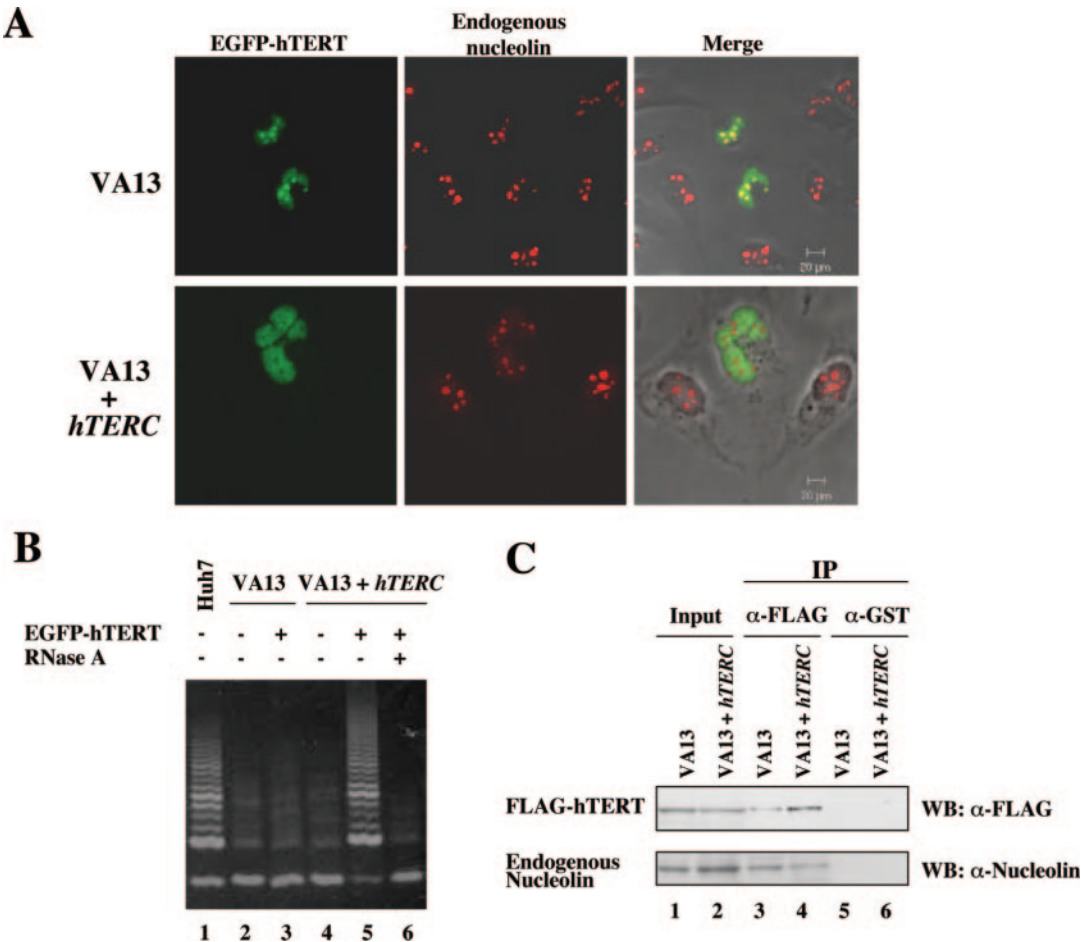


FIG. 7. *A*, distinct localization patterns of EGFP-hTERT in VA13 and VA13+hTERT cells. EGFP-hTERT was transiently expressed in VA13 or VA13+hTERT cells. Green, EGFP-hTERT; red, nucleolin; and yellow, merge. *B*, expression of EGFP-hTERT in VA13+hTERT but not in VA13 resulted in readily detectable telomerase activity. Lysates of VA13 and VA13+hTERT cells expressing (lanes 3 and 5, respectively) or not expressing (lanes 2 and 4, respectively) EGFP-hTERT were subjected to TRAP assay as in Fig. 5*A*. Huh7 cell lysate (lane 1) and RNase treatment of VA13+hTERT cells expressing EGFP-hTERT (lane 6) were used as a positive and a negative control, respectively. Input for each test tube was adjusted to $\sim 1 \times 10^3$ cells. *C*, hTERT interacts with endogenous nucleolin from both VA13 and VA13+hTERT cells. Total lysates of VA13 or VA13+hTERT cells expressing FLAG-hTERT were immunoprecipitated (IP) by α -FLAG antibody (lanes 3 and 4) and blotted using α -nucleolin or α -FLAG antibodies as indicated. Lanes 1 and 2 show input equal to 5%. α -GST antibody was used as a negative control (lanes 5 and 6).

telomerase complex to telomeres or by modulating the efficiency of telomerase RNP assembly. Several recent reports suggest that nucleoli and/or Cajal bodies are the sites for telomerase RNP assembly because both hTERT and hTERT are found in these structures (11–13, 16–18, 28, 29, 32, 33). Furthermore, a previous report showed that hTERT distribution between nucleoli and the nucleoplasm is regulated in a cell cycle-dependent manner in normal cells, and deregulation of the proper localization of hTERT in cancer cells correlates with the growth advantage observed in transformed cells (11).

In this report we demonstrate that nucleolin binds the active telomerase complex through both protein-protein and protein-RNA interactions. Two distinct regions of nucleolin are involved in the interaction with hTERT: a central region containing the RBD1 and a carboxyl-terminal region containing both the RBD4 and RGG domains. It remains unclear whether the region covering RBD1 binds hTERT alone or also weakly interacts with hTERT in telomerase. Although other protein-protein or protein-RNA interactions may also modulate these interactions, the observations indicate that nucleolin is a binding partner for telomerase.

Although the interaction of nucleolin and hTERT failed to alter telomerase activity *in vitro*, this interaction regulates the subcellular localization of hTERT. In this respect, these findings are reminiscent of a prior report that demonstrated

that the interaction of nucleolin and a virus RNA-dependent RNA polymerase (RdRP) altered the subcellular localization of RdRP (24). However, unlike the interaction of hTERT and nucleolin, the interaction of nucleolin with RdRP inhibited RdRP activity (24).

We show here that nucleolin affects subcellular localization of hTERT because the minimal hTERT binding region, nucleolin-4R, dramatically affected the subcellular localization of hTERT *in vivo*. Human TERT colocalizes with nucleolin-4R in the cytoplasm. Because the nucleolin-4R mutant lacks a nuclear localization signal, this protein-protein interaction of hTERT and nucleolin-4R is critical for the subcellular localization of telomerase.

Despite the distinct hTERT subcellular localization between normal and transformed cells, ectopically expressed hTERT appears to interact with endogenous nucleolin from both types of cell. These results suggest that the interaction between nucleolin and telomerase occurs not only in nucleoli but also in the nucleoplasm. One possible explanation for these findings is that further post-translational modifications of nucleolin or hTERT may facilitate nucleoplasmic localization of hTERT in cancer cells. Alternatively, other proteins may differentially modify the localization of the nucleolin-telomerase complex in normal cells and cancer cells. In either case, nucleolin appears to be involved in assembly or maturation steps of telomerase

because subcellular localization of hTERT was critically affected by the presence or the absence of *hTERC* as demonstrated in *hTERC*-negative VA13 cells. The nucleolar localization of hTERT in VA13 cells was not an intrinsic feature of ALT cells because in another ALT cell line, GM847, which expresses *hTERC*, hTERT was found to be preferentially localized to the nucleoplasm (11, and data not shown). Coexpression of EGFP-hTERT and GST-nucleolin-4R also dramatically changed cellular localization of EGFP-hTERT to the cytoplasm in hTERT-expressing VA13 cells as was observed in the Huh7 cells. In contrast, the nucleolar EGFP-TERT in VA13 cells was weakly affected by expression of GST-nucleolin-4R (Supplemental data, Fig. S2). The result suggests that the nature of nucleolin and the hTERT complex was affected by the presence of *hTERC*.

Nucleolin as an RNA chaperone complexed with hTERT may facilitate assembly of telomerase in the presence of *hTERC*. Such nucleolin-telomerase complexes are then exported from nucleoli to the nucleoplasm in a process that may involve masking of a nucleolar retention signal of hTERT and/or nucleolin. Other factors appear to regulate this process because overexpression of full sized nucleolin resulted in nucleolar colocalization of telomerase in cancer cells. The functional consequences of the association of nucleolin with telomerase in the nucleoplasm remain obscure. One possibility is that nucleolin maintains telomerase in the nucleoplasm ready for the delivery to telomeres. Nucleolin thus not only helps assemble telomerase but also provides a reservoir of telomerase ready for delivery to the telomere. Because telomerase is present in different subnuclear compartments in normal and malignant human cells, these observations suggest that alterations in telomerase assembly and localization play additional roles in cell transformation.

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Nucleolin Interacts with Telomerase

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