In vitro Regulation of Activation with Human Telomerase Reverse Transcriptase Components Expressed in *Escherichia coli* and Human Telomerase RNA Component

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Telomerase is a ribonucleoprotein (RNP) complex that elongates telomeres.[1,2] The telomere is a specialized structure at the ends of linear eukaryotic chromosomes that provides mechanism for maintaining chromosome length and has critical functions in maintaining chromosome stability.[1,3] The telomere contains distinctive repeats of guanidine-rich sequences (TTAGGG) that are replicated by DNA-dependent DNA polymerase and by telomerase-dependent synthesis of telomeric DNA from an RNA templates.[4] In a variety of organisms, the loss of telomerase activity results in telomere shortening and ultimately a period of cell death or growth arrest.[5] In human, the telomerase plays an important role in cancer. Most somatic cells lack telomerase activity and correspondingly lose telomeric DNA, thus are limited their proliferative capacity. Cancer cells can overcome this proliferative block through the illegitimate transcriptional up-regulation of the gene encoding the catalytic subunit of the enzyme.[6] Human telomerase is minimally composed of two subunits; the human telomerase reverse transcriptase (hTERT) identified as the catalytic enzyme required for telomere elongation and the human telomerase RNA components (hTR).[7-11] Recently, some groups reported *in vitro* reconstitution of telomerase and demonstrated the essential role of the hTERT and the hTR.[12-14] One group demonstrated that Hsp90 and p23 were essential for telomerase activity with recombinant hTERT synthesized *de nove* in rabbit reticulocyte extract *in vitro*.[14] Another group demonstrated that the production of the active recombinant telomerase of *Tetrahymena* requires a factor in rabbit reticulocyte extract that promotes ribonucleoprotein assembly. In these systems, certain factors carried over with rabbit reticulocytes may influence the native telomerase activity.[15,16] Therefore, it remains unclear whether these two components, the hTERT and the hTR are sufficient for *in vitro* telomerase reconstitution. Although the hTERT and the hTR are essential for telomerase activity, little is known about how telomerase is activated and maintained at the enzyme levels in human cancers. Some possible mechanism is that tertiary and quaternary structures of the large telomerase complex are modulated by protein phosphorlyation in such a way that the enzyme is activated.[17,18] The reconstitution of the hTERT, the hTR and the phosphorylation of the hTERT is necessary to solve question for telomerase activity and to provide an experimental system in which to identify factors that are essential for or that stimulate telomerase activity *in vitro*.

Here, we reported a method of *in vitro* regulation of activation with the hTERT expressed in *E. coli* and the hTR. Moreover, we demonstrated that the regulation of telomerase activity and subsequent maintenance of its activity required a prerequisite phosphorylation of the hTERT by protein kinase Cα.

The hTERT had not been reported expression and purification by *E. coli* expression system. We thought that the *E. coli* expression system was unsuitable for the hTERT expression because of different codon usage in eukaryotic expression system. Thus, the complete gene encoding the $hTERT_{1-1132}$ was divided into three parts ($hTERT_{1-397}$, $hTERT_{397-676}$ and $hTERT_{676-1132}$). The primers for the amplification of the each hTERT fragment were designed by using *E. coli* codon usage (Table 1). The constructed hTERT gene encoded $hTERT_{1-1132}$ was transformed into *E. coli* strain BL21 Star (DE3) contained pACYC-based plasmid encoding the *argU* and *proL* tRNA and pSC101-based plasmid encoding the *argU*, *ileY*, and *leuW* tRNA. The expression level of the recombinant 6xHis-tagged hTERT in BL21 Star

Table 1. Primers for the construction of the hTERT expression vector

Name	Direction	Sequence	Restriction enzyme site
$hTERT_{1-397}$	sense	5'-GAATTCATGCCGCGTGCTCCGCGCTGCCGAGCC-3'	*Eco*R I
	anti-sense	5'-CAAGGAGCTCCAGAAACAGCGGACGCATCTGCC-3'	*Sac* I
$hTERT_{397-676}$	sense	5'-CTGGAGCTCCTGGGTAACCACGCGCAGTGCCC-3'	*Sac* I
	anti-sense	5'-CACGTCGACCTTGACAAAGTACAGTTCCGGCGG-3'	*Sal* I
$hTERT_{676-1132}$	sense	5'-AAGGTCGACGTTACGGGTGCGTACGACACCATCC-3'	*Sal* I
	anti-sense	5'-AAGCTTATCAGTCCAGGATGGTCTTGAAGTC-3'	*Hind* III

(DE3) contained the two plasmids encoding tRNA was much higher than that in BL21 Star (DE3). For the more efficient expression of the recombinant protein, we therefore selected the BL21 Star (DE3) contained the two plasmids as an expression host. The amount of the expressed 6xHis-tagged hTERT was almost similar to during culture for 6h after the inoculation of 0.1 mM or 1.0 mM IPTG at OD_{600} = 0.6-0.7 or 0.9-1.0 as judged by coomassie-stained 6% SDS-PAGE. However, the expression level of the recombinant 6xHis-tagged hTERT for 6 h culture after inoculation of 0.1 mM IPTG at OD_{600} = 0.6-0.7 was much higher than other expression condition as judged by Western-blotting analysis using telomerase antibody (Figure 1A and 1B). The recombinant the 6xHis-tagged hTERT was expressed as a soluble form in contrast with recombinant FLAG-hTERT in insect cell.[19] However, the 6xHis-tagged hTERT was eluted with other proteins at lower imidazole concentration under native condition. Therefore, we attempted to purify the 6xHis-tagged hTERT under denaturation condition using 8 M urea, and then identified the band with an apparent monomer mass of ~130 kDa as determined by 6% SDS-PAGE (Figure 1C). These results strongly suggested that the 6xHis tag at *N*-terminus of the hTERT was not exposed in native tertiary structure but was exposed under denaturation condition. The 6xHis-tagged hTERT was isolated in a yield of approximately 0.1 mg per liter of cultures.

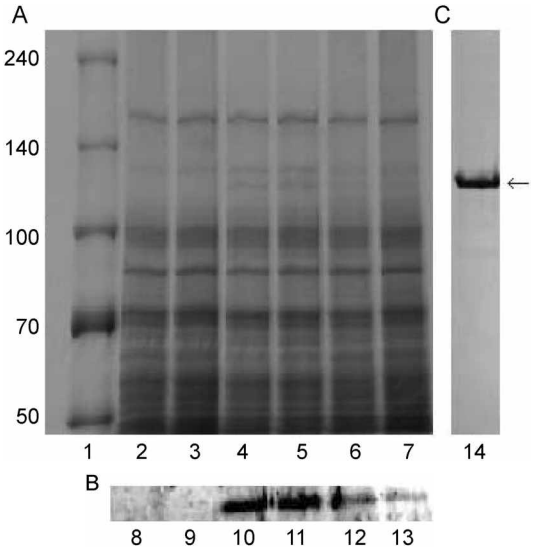

Figure 1. 6% SDS-polyacrylamide gel electrophoresis analysis and Western-blotting analysis of the 6xHis-tagged hTERT by *E. coli*. A, lane 1, Molecular weight standard marker; lane 2, Supernatant of *E. coli* containing pET-28a(+) expression vector; lane 3, Supernatant of *E. coli* containing plasmid encoding the hTERT without IPTG; lane 4, 5, 6 and 7, Supernatant of *E. coli* containing plasmid encoding the hTERT with IPTG. B, Soluble fractions as described in A were subjected to Western-blotting analysis with the hTERT antibody (lane 8, Supernatant of *E. coli* containing pET-28a(+) expression vector; lane 9, Supernatant of *E. coli* containing plasmid encoding the hTERT without IPTG; lane 4 and 10, OD_{600} = 0.6-0.7, 0.1 mM IPTG; lane 5 and 11, OD_{600} = 0.6-0.7, 1.0 mM IPTG; lane 6 and 12, OD_{600} = 0.9-1.0, 0.1 mM IPTG; lane 7 and 13,OD_{600} = 0.9-1.0, 1.0 mM IPTG) C, lane 14, The concentrated hTERT protein after purification under denaturing condition.

Although the phosphorylation sites on the hTERT have not been determined yet, protein kinase Cα in human breast cancer cells is required for the activation of telomerase activity and subsequent maintenance of its activity.[18] We attempted the phosphoylation of the purified 6xHis-tagged hTERT by protein kinase Cα (Figure 2). When the hTERT was not phosphorlated by protein kinase Cα, telomerase activity was not detected. This result indicates that the phosphoylation of the hTERT must be required for telomerase activation. However, we do not know how the phosphorylation is regulated by kinase, depending on the cell cycle or the outside signal. After the phosphorylated 6xHis-tagged hTERT and the transcribed hTR *in vitro* were reconstitutied, telomerase activity was detected qualitatively and quantitatively by using telomerase assay kits. Telomerase activity was detected only when the phosphorylated hTERT and the hTR were present, although the phosphorylated hTERT alone did not exhibit telomerase activity (Figure 2). Also, the telomerase activity of the *in vitro* reconstituted components was measured quantitatively in the presence of varying molar ratios of the phosphorylated hTERT and the hTR. Maximum telomerase activity was observed when the phosphorylated hTERT and the hTR were present at approximate equal molar ratio in the reaction mixture. Judging from the results obtained by these two methods, the reconstitution of equal molar amounts of the phosphorylated hTERT and the hTR seemed to occur resulting in the optimal telomerase activity, suggesting efficient complex formation of the components *in vitro*.

In summary, we constructed expression plasmids suitable for the hTERT expression using *E. coli* usage codon and expressed as a soluble form in *E. coli* expression system. The activated hTERT by the phosphorylation of specific sites and the hTR are minimal components for telomerase activity *in vitro*. This expression system, the purification method and the regulation method of telomerase activity for the human telomerase will be useful not only for the mechanism and structural studies of telomerase but also for the developments of drug design targeting the hTERT in anticancer therapy.

Experimental Section

Materials. The pET-28a(+) expression vector, *E. coli* strain BL21 Star (DE3) star and His-bind resin used in this study were supplied from Novagen, Inc. (Madison, WI, USA). Restriction enzymes, DNA-modifying enzymes, ATP and dNTP were obtained from Takara Shuzo, Co. Ltd. (Otsu, Shiga, Japan). The synthesis of DNA primers was performed by Cosmo genetech (Seoul, Korea). Gel electrophoresis reagents and protein assay reagents were from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). MEGAscripts (T7) and RNase inhibitor were from Amion, Inc. (Austin, USA). Amiconultra-15 (MWCO: 30000) and Microcon YM-50 were from Amicon, Inc. (Beverly, MA, USA). Protein kinase Cα, Akt protein kinase and Abl protein tyrosine kinase were from Upstate Biotechnology, Inc. (Lake Placid, New York,

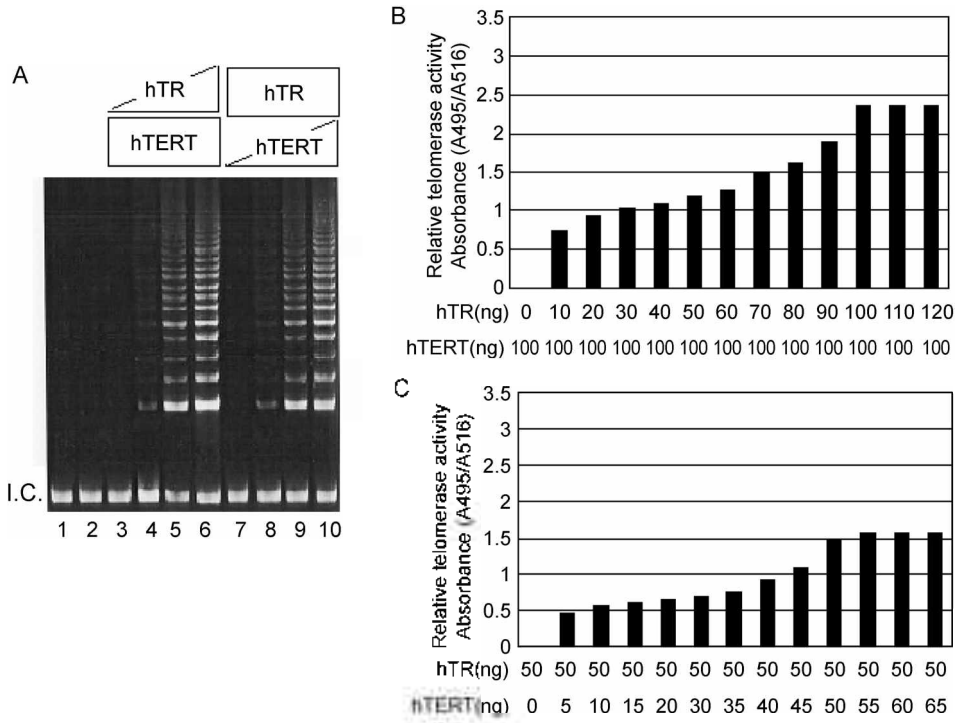

Figure 2. Regulation of telomerase activity with the hTERT activated by protein kinase Cα and the hTR reconstituted *in vitro*. A, Telomerase activity was measured by TRAPeze telomerase detection kit (Gel-base TRAP assay). lane 1, as a negative control, telomerase reaction was performed with only telomerase storage buffer (buffer A); lane 2, 100 ng of the hTR and 100 ng of the non-phosphorylated 6xHis tagged hTERT were subjected to the reaction mixture for telomerase activity; lane 3-6, telomerase reaction was performed with 100 ng of the phosphorylated 6xHis tagged hTERT by protein kinase Cā and of 0, 30, 60 and 100 ng of the *in vitro* transcribed hTR; lane 7-10, 50 ng of the *in vitro* transcribed hTR, and 0, 15, 30 or 50 ng of the phosphorylated 6xHis tagged hTERT by protein kinase Cα. B, The relative telomerase activity was measured by TRAPeze XL telomerase detection kit under varying amounts of the hTR at constant amounts of the phosphorylated 6xHis tagged hTERT. C, Under varying amounts of the phosphorylated 6xHis tagged hTERT at constant amounts of the hTR.

USA). TRAPeze Telomerase Detection Kit and TRAPeze XL Telomerase Detection Kit were from Chemicon International, Inc. (Temecula, CA, USA). All other chemicals and reagents were used the highest reagent grade commercially available from Sigma-Aldrich (St. Louis, MO, USA).

Plasmid construction for the hTERT expression. Each plasmid for the hTERT expression was made by sub-cloning of the *Eco*RI-*Sal*I fragment of FLAG-hTERT cDNA. For the construction of the hTERT expression plasmid, each hTERT fragment contained proper restriction enzyme site was amplified by polymerase chain reaction (PCR) with the six oligonucleotide primers (Table 1). The each resultant PCR products were subcloned into yT&A cloning vector (Real-Biotech) using TA cloning methods and confirmed to be correct by direct sequencing. Then, the *Sac*I site contained third hTERT fragment ($hTERT_{676-1132}$) was removed by site-direct mutagenesis with the following primers 5'-GTCGTCATCGAGCAGTCCTCCTCCCTGAATG-3' and 5'-CATTCAGGGAGGAGGACTGCTCG ATGACGAC-3'. The yT&A containing the each hTERT fragment was digested by *Eco*RI/*Sac*I, *Sac*I/*Sal*I and *Sal*I/*Hind*III, and inserted into the same restriction enzyme sites in pET-28a(+) one after the other. As a result, the hTERT gene coded $hTERT_{1-1132}$ was cloned completely and used to transform into *E. coli* strain BL21 Star (DE3) contained ColE1-compatible, pACYC-based plasmid coded the *argU* and *proL* tRNA and a ColE1- and pACYC-compatible pSC101-based plasmid coded the *argU*, *ileY*, and *leuW* tRNA.

Expression and purification of the 6xHis-tagged hTERT. *E. coli* strain BL21 Star (DE3) harboring the constructed plasmid was grown in LB broth containing kanamycin (50 μg/mL), chloramphenicol (34 μg/mL) and streptomycin (75 μg/mL) at 37 °C until OD_{600} = 0.6-0.7, and the recombinant protein was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma) for 6 h.[20,21] The induced cells were harvested by centrifugation at 10,000 g for 10 min at 4 °C then, resuspended in 20 mM Tris-HCl buffer, pH 7.9, containing 100 μM phenylmethanesulfonyl fluoride (Sigma) and lysised with as ultrasonic processor (Sonics and Materials) at 4 °C for 10 min. After centrifugation (12,000 g, 30 min), a soluble fraction of the extract containing the 6xHis-tagged hTERT was judged by coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western-blotting using hTERT antibody (Abcam). The supernatant was added 8 M urea and incubated at room temperature for 30 min and purified by His-Bind resin under denaturation condition. The supernatant was washed with 20 mM Tris-HCl containing 0.5 M NaCl, 20 mM imidazole and 6 M urea, pH 7.0, and the enzyme was eluted with 20 mM Tris-HCl containing 0.5 M

NaCl, 40 mM imidazole and 6 M urea, pH 7.0. The pool of active fractions was dialyzed against buffer A (20 mM Tris-HCl containing 3 mM $MgCl_2$, 6 mM KCl, 1 mM EGTA and 1 mM DTT, pH 7.5) and then was concentrated by Amicon-ultra-15 (MWCO: 30000).

Preparation of the hTR. The gene encoding hTR (Genebank number, U86046) was generated by PCR from HeLa genomic DNA with the following two primers. The sequences of primers used in PCR were 5'-GGGCCCGGGTTGCGGAGGGTGGGCCTGGGAG-3' (*Apa* I) and 5'-GAGCTCGCATGTGTGAGCCGAGTCCTGGGTGC3' (*Sac* I). The resultant PCR product was digested by *Apa* I and *Sac* I and inserted into the plasmid vector pGEM-T easy (Promega) containing the T7 RNA polymerase promoter site. The resultant vector, pGEM-hTR was used to transform into *E. coli* strain XL1-blue (Stratagene). The hTR was prepared with the MEGAscripts (T7) kit using pGEM-hTR digested by *Ecl*136II (neoschizomer of *Sac* I, Fermentas). The transcription reaction was treated with 3U RNase-free DNase per μg of DNA at 37 °C for 15 min and purified by denaturing polyacrylamide gel.

***In vitro* phosphorylation of the 6xHis-tagged hTERT.** Protein kinase Cα was activated with the protocol provided by the manufacture. The reaction mixtures were allowed to proceed at 30 °C for 30 min and then performed to exchange phosphorylation buffer for buffer A by Microcon YM-50 to measure telomerase activity.

Telomerase activity measurement. The appropriate amount of the *in vitro* transcribed hTR and the phosphorylated 6xHis-tagged hTERT were mixed together, incubated at 33 °C for 10 min for reconstitution, and then subjected to telomerase reaction. Telomerase activity was measured using a commercially available PCR-based according to the manufacturer's protocol.[10,22] After PCR amplification, PCR products were resolved on 12% non-denaturing polyacrylamide gel for qualitative analysis and then visualized by staining with ethidium bromide (Sigma) and measured by fluorescence for quantitative analysis.

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