Identification of telomere binding region and ribozyme target site in human telomerase RNA

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Identification of telomere binding region and ribozyme target site in human telomerase RNA

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This certifies that the Doctorial Dissertation of Marie Yeo is approved.

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The Graduated School
Yonsei University

December 2002

감사의 글

3년의 학위과정동안 힘들고 기쁜 일들을 함께하며 학위를 마무리 할 수 있도록 도와주신 여러 선생님과 가족들에게 감사의 마음을 전합니다.

본인의 실험 외에도 여러 연구분야를 항상 접할 수 있는 기회를 주시고 물심양면으로 도와주신 정현철 지도 교수님께 먼저 감사의 말씀을 전합니다.

학위논문에 많은 조언으로 자문을 해주신 노재경 교수님, 노성훈 교수님, 아주대 임호영 교수님, 고려대 김준석 교수님께 감사를 드립니다.

실험실 생활에 세심한 배려를 아끼지 않으신 라선영, 남석우 교수님과 오랜 시간 동안 함께 정을 나눈 실험실 많은 식구들께 다시 한번 감사 드립니다.

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끝으로 학위 하느라 소홀한 아내의 자리를 말없이 이해해주고 감싸준 사랑하는 남편 정훈씨께, 건강하게 태어나 엄마로서 행복을 느끼게 해준 아들 찬휘에게 가슴깊이 사랑과 감사의 말을 전합니다. 언제나 주신 사랑과 은혜를 갚을 길이 없는 사랑하는 아빠, 엄마께 이 작은 성과를 바칩니다.

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Human telomerase RNA (hTR), an essential component of telomerase, has unique secondary structure consisted of several distinct structural domains as well as template sequence which directs telomeric DNA synthesis at the ends of chromosomes. It is well established that hTR has to be accessible for both telomere and telomerase catalytic subunit (hTERT) for the specialized reverse transcriptase activity of telomerase. Previous studies suggested that only small fragment (159 nucleotides) of hTR is sufficient for the interaction with hTERT, but

structural domains of hTR required for interaction with telomere have not been elucidated. Here, we report that at least, the 326 nucleotides fragment of hTR consisted of the template, pseudoknot, and CR4-CR5 domain is required to exhibit a binding capacity with telomere, and simultaneously display a telomerase activity *in vitro* reconstitution assay. Furthermore, targeted disruption of the template region with antisense oligonucleotide results in the complete blocking of the hTR-telomeric DNA binding, whereas CR4-CR5 disruption totally abrogate telomerase activity *in vitro.*

Since the reactivation of telomerase is thought to be an essential step in immortalization of cells and tumor progression, it is suggested as a good target for anti-cancer strategies. Specific inhibition of telomerase in tumor cells leading to telomere shortening, and eventually, cell death has been demonstrated in recent papers. Ribozyme is a RNA molecule which possesses specific endoribonuclease activity and catalyzes the hydrolysis of specific phosphodiester bonds resulting in the cleavage of the RNA target sequences. Here, we evaluated a target site of hammerhead ribozyme in human telomerase RNA to inhibit telomerase activity. Hammerhead ribozyme targeting the template region of hTR efficiently cleaved human telomerase RNA *in vitro.* Stable transfectants of ribozyme

showed degradation of hTR, and diminished telomerase activity. Moreover, ribozyme transfectants displayed significantly delayed doubling time than parental cells.

In conclusion, we demonstrated that hTR interacted with telomeric DNA through a 326 nucleotides of hTR including template, pseudoknot, and CR4-CR5 domains with distinct roles in terms of telomere binding and telomerase activity. The template region of hTR was the best target site for inhibition of telomerase activity, as anticancer strategy using ribozyme in human breast tumor cell.

Key Words: Telomere, Telomerase, Human Telomerase Protein Component hTERT, Human Telomerase RNA Component hTR, Cancer, Ribozyme.

Identification of telomere binding region and ribozyme target site in human telomerase RNA

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I. INTRODUCTION

Telomerase is a specialized reverse transcriptase that replenishes telomere through the adding of tandem repeats (TTAGGG) of telomeric DNA onto the ends of chromosomes[1, 2]. Telomeres are necessary for eukaryotic chromosome stability and the complete replication of chromosome end [3, 4, 5]. The termini of telomeric DNA cannot be fully replicated by the end replication problem of DNA polymerase, thus a net loss of telomere sequences occurs at each cell division [6,7]. It has been proposed that this cumulative telomeric erosion is a

limiting factor in replicative capacity and elicits a signal for the onset of cellular senescence [8, 9]. To proliferate beyond the senescence checkpoint, cells must restore telomere length. This can be achieved by telomerase, which is inactivated in most normal somatic cell, but is reactivated in most cancer cells [10, 11]. Thus, telomerase plays an important role in both cellular senescence and especially in immortalization of transformed primary cells due to its pivotal role in unlimited cellular proliferation (Figure 1) [12].

The current evidence of a strong correlation between telomerase activation, cellular immortalization, and cancer clearly identifies both telomere and telomerase as specific targets for anti-cancer therapy [13, 14]. Table 1 showed the various approaches to telomerase inhibition that are recently under investigation. Specifically, taking advantage of structural and functional similarities in telomerase and retroviral reverse transcriptase, reverse transcriptase inhibitors, such as nucleoside analogs, have been also used as inhibitor of telomerase activity [15, 16]. An additional targeted strategy dealt with components of telomerase, hTERT and hTR. In fact, it has been shown that chemically modified oligonucleotides, such as phosphorothioate oligomers, peptides nucleic acids and hammerhead ribozymes targeting telomerase components reduced telomerase activity of tumor cells [17, 18, 19].

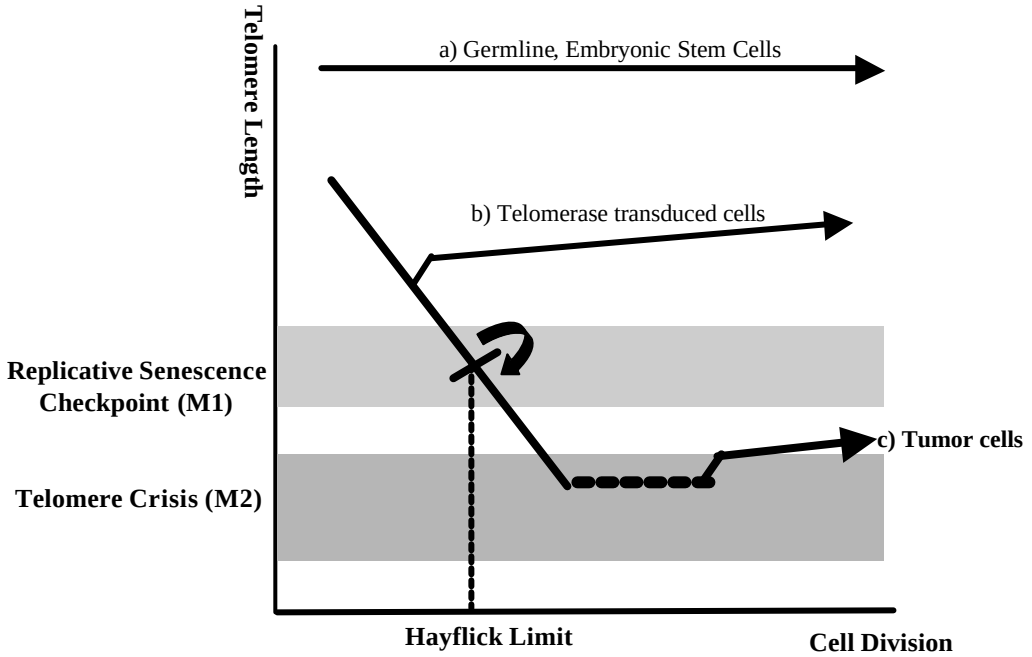

Figure 1. Schematic figure of the telomere hypothesis of cellular aging and immortalization based on Harley *et al.* [12]. The relationship between telomere length and cell divisions in normal somatic cells and tumor cells. In normal somatic cells, there exists at least two telomere-dependent mortality phases (M1 and M2). Replicative senescence is characterized by a checkpoint arrest trigged by a DNA damage response due to critical telomere loss. If cells lack this checkpoint, they can continue to divide, losing telomeric DNA until the crisis phases characterized by major telomere dysfunction, genetic instability and apoptosis. It illustrates telomere maintenance in immortal germline (a), in normal somatic cells immortalized by etopic expression of telomerase (b), and in tumor cells which have undergone growth control mutations and abnormal reactivation of endogenous telomerase (c).

Table 1. Approaches for targeting telomerase in cancer therapy

Component	References
Telomerase RNA component	
Antisense [23, 24]	Feng *et al*., 1995; Kondo *et al*., 1998
2-5A antisense [17, 19]	Kondo *et al*.,1998, 2000
Ribozyme [20, 21]	Yokoyama *et al*., 1998; Folini M *et al*., 2000
* PNA [25, 26]	Norton *et al*., 1996; Shammas *et al*., 1999
Telomerase catalytic subunit	
Dominant negative hTERT [27, 28]	Hahn *et al*, 1999; Zhang *et al*., 1999
Reverse transcriptase inhibitor [29, 30]	Strahl and Blackburn, 1996; Melana *et el*., 1998
Ribozyme [31, 32]	Yokoyama *et al*., 2000; Ludwig *et al*., 2001
Reverse transcriptase inhibitor [15, 16]	Gemez *et al*,1998; Stranhl *et al*, 1996
G-quadruplex stabilizer [42, 43]	Han *et al*, 2000; Kerwin *et al*, 2000

*PNA; Peptide Nucleic Acid

Ribozymes are small catalytic RNA molecules possessing specific endonuclease activity. The catalytic sequences can be designed to specifically cleave a target RNA sequences by incorporating the flanking sequences complementary to the target. They are being increasingly considered and used as human gene therapeutic agent against human malignancies [20, 21, 22].

The synthesis of telomeric DNA requires at least two components of telomerase: the protein catalytic subunit (hTERT) and the RNA subunit (hTR) (Figure 2). The RNA component of telomerase contains a short template sequence that specifies the telomere repeat, which is also essential for telomerase enzymatic activity. Recently, a secondary structure of the human telomerase RNA has been proposed based on a phylogenetic comparison analysis, and revealed that it contains several structural domains conserved in all vertebrates implying an evolutionary conservation of telomerase RNA [33]. The conserved secondary structures are composed of several distinct domains including the template, pseudoknot, CR4-CR5, Box H/ACA, and the CR7 domains (Figure 3, A). This finding suggested that a conserved structure of hTR may play an essential role in the regulation of telomerase activity.

Human telomerase activity can be reconstituted by the addition of *in vitro* transcribed hTR to rabbit reticulocyte lysates (RRL) expressing hTERT [34], MNase treated cancer cell lysate [35] or recombinant hTERT purified from

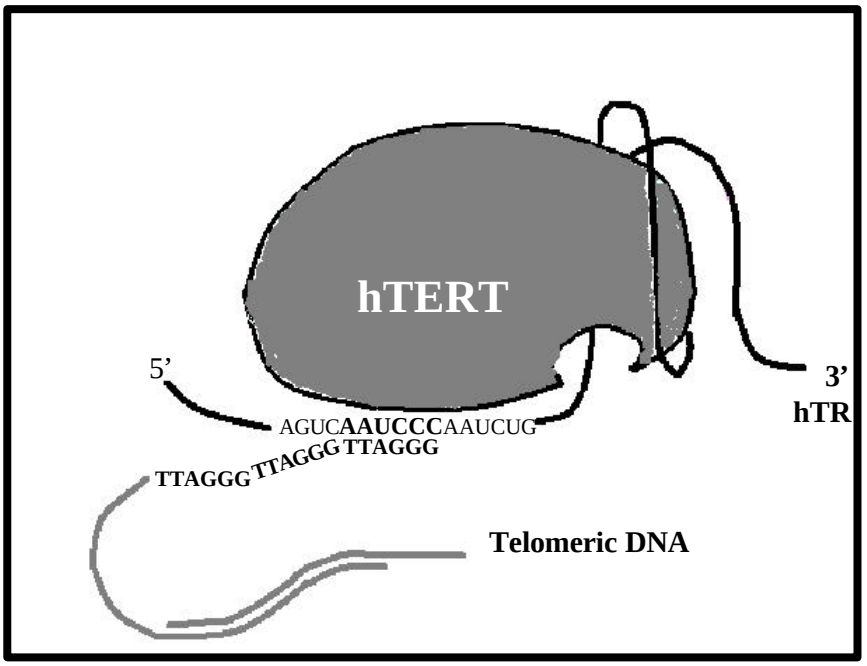

Figure 2. A model of human telomerase RNP assembly. Telomerase is a ribonucleoprotein (RNP) composed of protein subunit hTERT and RNA subunit hTR. The large one represents hTERT catalyzing new telomere synthesis through the reverse transcription of template sequences of hTR. The RNA component hTR containing the template sequences interacts with both hTERT and end of telomere. The possessive cycles of telomere binding, elongation, and translocation add TTAGGG repeats to the 3' telomeric terminus.

baculovirus–infected insect cells [36]. *In vitro* assembly reactions using deleted or site-directed hTR mutants suggested that the structural domains played critical roles in telomerase activation. Wright *et al.* reported that a minimal region required for the expression of telomerase activity is the nucleotides between +33-+325 [37]. Autexer *et al.* suggested a direct interaction between hTR and hTERT, based on the fact that 159 nucleotides of hTR were sufficient for the stable interaction with hTERT [38]. The CR4-CR5 and pseudoknot domains have also been suggested as critical domains for the reconstitution of telomerase activity [39, 40]. However, significance of each structural domain of hTR in binding with telomeric DNA and effect of this binding on telomerase enzymatic activity had not been elucidated.

In this study, we present the minimal structural lengths of hTR that confers the ability to make hTR-telomeric DNA complex *in vitro* as well as the reconstitution of telomerse activity. To assess minimal structural region of hTR, we generated truncated hTR mutants based on the functional domains of hTR secondary structure. These mutants were then tested for the ability of binding with telomeric DNA utilizing modified electrophoretic mobility shift assay (EMSA) with single stranded telomeric repeat sequences, (TTAGGG)n. The telomere binding capacity of each mutant was also directly compared to its reconstitutional ability of telomerase activity and hTERT binding ability.

In order to make a complex of telomeric DNA and hTR, the results showed that hTR required the its major functional regions comprised the template, pseudoknot and CR4-5 domains. Moreover, we also found that each mutants displayed telomerase activity parallel to the binding capacity of the same mutant implying that the minimal hTR length contributes to both telomeric DNA binding and telomerase activation. Functional study using antisense oligonucleotide also suggested that there might be independent regulatory regions in terms of telomeric DNA binding and activation of telomerase on hTR structural regions by the results that targeted disruption on template region efficiently abrogated telomeric DNA binding and telomerase activity. However, targeted inhibition of CR4-5 region only exhibited the loss of telomerase activity.

Based on the *in vitro* study results, we evaluated the ability of a hammerhead ribozyme targeted the hTR to inhibit the catalytic activity of telomerase. We verified the ribozyme specificity by means of its ability to cleave the synthetic RNA substrate. Subsequently, we examined whether the stable expression of ribozyme targeted the hTR inhibited telomerase activity, which resulted in growth retardation of breast tumor cell, MCF-7.

Ⅱ. MATERIALS AND METHODS

1. Identification of telomere binding region in human telomerase RNA

A. Preparation of hTR

To investigate the role of the structural domains in human telomerase RNA, we generated truncated hTR mutants based on the proposed secondary structure of hTR [33]. Full length or truncated mutants were obtained by PCR amplification of HT1080 genomic DNA with each oligonucleotides primers encoding the sequences of each domain of hTR as follows: D1 (+1-+183), (+1-+212), D3 (+1-+326), D4 (+1-+363), D5 (+1-+407), D6 (+1-+451), D7 (+64-+451). The PCR products were cloned into pGEM-T vector (Promega) containing the T7 RNA polymerase promoter sequence at the 5' end of the multiple cloning region. Full length or truncated mutants of hTR were transcribed *in vitro* using the RiboProbe transcription system (Promega) (**Figure 3 B**).

B. Preparation of hTERT

The full-length cDNA of hTERT was kindly provided by Dr. S.C. Kim (Genoprot. Korea). Insert DNA containing the entire hTERT coding sequences

fused with a HA-epitope tag at C-terminal was subcloned into pCI neo vector at the sites of *EcoR* I – *Sal* I. hTERT-HA protein was synthesized by using a rabbit reticulocyte lysate transcription / translation system (TNT® System; Promega) according to the manufacturer's instructions. In brief, hTERT-HA cDNA was added to rabbit reticulocyte lysate (RRL) at a concentration of 0.04 μg/ μL and incubated at 30°C for 90 min. The translation product was used for the *in vitro* telomerase reconstitution.

C. *In vitro* telomerase reconstitution

Human telomerase was reconstituted in 5 μL of reaction mixture containing 150 ng of *in vitro* transcribed hTR, 2 μL of *in vitro* synthesized hTERT in RRL and 2 μL of fresh RRL. The mixture was incubated at 30°C for 90min and diluted by 10-fold in CHAPS lysis buffer (10mM Tris-Cl, pH7.5, 1mM $MgCl_2$, 1mM EGTA, 0.1mM PMSF, 0.5% CHAPS, 10% glycerol). Five micro liters of this mixture was used for the telomerase enzyme assay.

To determine the effect of antisense oligonucleotide on the assembly of active telomerase, 50 pmoles of oligonucleotide were incubated with the hTR at 30°C for 15 min prior to the addition of *in vitro* synthesized hTERT

D. TRAP assay

The TRAP assay was performed as described by Kim *et al*. with some modifications [41]. Briefly, for the extension of TS primer (5'-aatccgtcgagcagagtt-3'), 5 μL of reconstituted telomerase was added to a reaction mixture containing 62.5 μM dNTP, 5 ng/μL TS, 125 μg/ml BSA in TRAP buffer (5mM Tris-Cl, pH 8.3, 0.37mM $MgCl_2$, 15mM KCl, 0.00125% Tween 20, 0.25mM EGTA). After incubation at 30°C for 60min, the sample was subjected to two-step PCR (94°C, 30s and 60°C, 30s) for 28 cycles with 5 X 10^{-21} M TSNT (5'-aatccgtcgagcagagttaaaaggccgagaagcgat-3'), 0.2 ng/μL NT (5'-atcgcttctcggcctttt-3'), 2 ng/μL ACX (5'-gcggcggcttacccttacccttacccta -3'), 62 uM dNTP, 0.16 μCi/ μL (3000Ci/mmole, Amersham Pharmacia, Piscataway, NJ), 2.5U Taq polymerase (GiBCO BRL, Grand Island, NY) in PCR buffer (1.5mM $MgCl_2$, 20mM Tris-Cl, pH 8.4, 50mM KCl). The PCR product was resolved on 12% polyacrylamide gel at 340 V for 3hrs, and the gel was dried and visualized by autoradiography. The relative activity of the telomerase was determined using the following formula. TPG (%)=Sample [(T-B)/ CT]÷wild type [(T-B)/ CT] X 100 (TPG; telomerase activity, T; total intensity of telomerase-mediated bands, B; intensity of the negative control, CT; intensity of the internal control).

E. EMSA

To identify the binding ability of the hTR mutants to telomere, EMSA was performed by using ^{32}P-labeled telomeric sequences, (TTAGGG)n. In standard binding reactions, 150 μg of the hTR mutants was incubated with 1pmole of end labeled $(TTAGGG)_3$ in 1x binding buffer (50mM Tris-Cl, pH 7.5, 5mM $MgCl_2$, 1mM EDTA, 50mM NaCl, 1mM DTT, 5% glycerol, 66ng/μL poly[dI.dC]) at 37°C for 30 min. The binding complex was irradiated with 15mJ in XL-1500 UV cross-linker (Spectronic corporation, New York, USA) for 5min. Two micro liters of 80% glycerol were added to the 8 μL of the reaction mixture, and electrophoresis was conducted in a 1.5 % agarose gel or 6% polyacrylamide gel in 0.5X TBE buffer (89mM Tris-borate, 2mM EDTA) at 4°C for 4-5 hrs at 80 V.

To determine the effect of antisense oligonucleotide on complex formation, 100-fold molar excesse of unlabeled oligonucleotide was added to the reaction mixture prior to adding labeled probe and incubated at 37°C for 15 min. Primer sequences used for the antisense analysis were as follows: 5'-tcagttagggttagac-3' for the template domain, 5'-gtttgctctagaat-3' for the pseudoknot domain, and 5'-gctgacagagcccaact-3' for the CR4-CR5 domain. Antisense oligonucleotide was designed to target the unpaired region in the functional domain of hTR based on the proposed secondary structure by Chen

et al. (**Figure 3 A**).

F. Immunoprecipitation

Fifty microliters of assembly reaction mixture was precipitated by adding anti-HA mouse monoclonal antibodies (Santa Cruz Biotechnology, Inc) and 70ul of protein G agarose (Santa Cruz Biotechnology, Inc) in IP buffer (50 mM HEPES, pH7.6, 100 mM NaCl, 0.1% NP-40, 0.2 mM EGTA, 1 mM MgCl2, and 10% glycerol). Agarose pellet was washed in washing buffer(20 mM HEPES, pH7.6, 20% glycerol, 10 mM NaCl, 0.2 mM EGTA, 1 mM $MgCl_2$, 0.1% NP-40, and 0.1% BSA). Two microliters, 10ul, and 30ul of beads were analyzed for TRAP, Western, and Northern analysis, respectively.

G. Western analysis

Assembly reaction mixtures or immunoprecipitants were electrophoresed onto 8% SDS-PAGE gel. The gel was transferred to PVDF membrane in a buffer (39mM glycine, 48mM Tris base, and 20% methanol). The membrane was blocked in 5% skim milk, probed with 1:1000 diluted anti-HA primary antibodies, and incubated with 1:2000 diluted horseradish peroxidase-conjugated secondary antibodies (Amersham). The membrane was visualized by ECL according to the manufacturer's instruction.

H. Northern analysis

The integrity and amount of hTR mutants were verified by northern hybridization analysis. To isolate RNA, assembly reaction mixtures were extracted with phenol: chloroform: isoamyl alcohol (25:24:1), and precipitated with 0.1 volumes of 3M sodium acetate, and 2.5 volumes of ethanol. The isolated RNA was electrophoresed onto 1.5% agarose–2.2M formaldehyde gel and transferred to a Hybond-N + membrane (Amersham). The membrane was probed with ^{32}P-labeled oligonucleotides complementary to hTR nt+164 to nt+183. The blot was washed twice for 30min at room temperature in 0.1x SSC/0.1% SDS buffer and performed autoradiography

2. Ribozyme target sites in human telomerase RNA

A. Ribozyme synthesis and *in vitro* cleavage assay

Hammerhead ribozymes were designed against seven NUX sequences (where N can be any ribonucleotides, and X can be any ribonucleotides excepting guanine) located in open loops on hTR secondary structure. Using four or five antisense nucleotides on each side of catalytic core, they designed to cleave 3' of the Cs at position 46, 156, 160, 275, 313, 317, and 360 of hTR. As shown figure 9, the ribozymes were named R1-7 from 5' end of hTR target

site and Rm (mis-matched ribozyme). The ribozymes for *in vitro* cleavage reaction were obtained by *in vitro* transcription of PCR product. A set of oligomers for PCR amplification was designed to make the ribozyme template containing T7 RNA polymerase promoter sequence at 5' upstream of ribozyme. One set of primer contained a T7 RNA polymerase promoter sequence followed by the 5' half of the ribozyme sequences; the other primer contained the antisense sequences of ribozyme. The primers used for each ribozyme were as follows: 5'-tgtaatacgactcactataggagggttactgatgagtccgtg-3'and 5'-tttttgtttcgtcctcacggactcatcagtaaccct-3'for R1; 5'-tgtaatacgactcactat aggtagaatctgatgagtccgtg-3'and 5'-caccgttttcgtcctcacggactcatcagattcta-3'for R2; 5'-tgtaatacgactcactatagggctctactgatgagtccgtg-3'and 5'-gttcattttcgtcctcacgg actcatcagtagagc-3'for R3; 5'-tgtaatacgactcactataggctgacactgatgagtccgtg-3'and 5'-gttgggctttcgtcctcacggactcatcagtgtcag-3'for R4; 5'-tgtaatacgactcactataggcg gctctgatgagtccgtg-3'and 5'-gctctgtttcgtcctcacggactcatcagagccg-3'for R5; 5'-tgtaatacgactcactataggcctccgctgatgagtccgtg-3'and 5'-ggcttctttcgtcctcacggactc atcagcggagg-3'for R6; 5'-tgtaatacgactcactatagggcctctgatgagtccgtg-3'and 5'-aggccttttttcgtcctcacggactcatcagaggc-3'for R7; 5'-tgtaatacgactcactataggtgaaaa actgatgagtccgtg-3'and 5'-ttccagtttcgtcctcaaggactcatcagtttttca-3'for Rm. The primer sets were mixed to form a hemiduplex, and a PCR amplification of 25 cycles was performed at 94 °C for 1 min, 40 °C for 1 min, and 72 °C for

1min. The ribozyme transcript was obtained by *in vitro* transcription of PCR product using a T7-Mega shortscript kit (Amion, Inc., Austin, TX).

To produce a synthetic RNA substrate, D6 were transcribed in the presence of a-^{32}P UTP using the RiboProbe *In Vitro* transcription system (Promega). The ribozyme and substrate RNA were mixed at 5:1 molar ratio in 10 ul reaction volume containing 50mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 1mM EDTA. The mixture was incubated at 28 °C overnight and stopped by the addition of equal volume of stop buffer. The reaction mixture was electrophoresed in a 6% polyacrylamide-7M urea gel. The gel was dried and visualized by autoradiography.

B. Construction of the ribozyme expression vector and transfection

Two single-stranded oligodeoxynucleotides were synthesized in a way that the 42bp ribozyme contained franking *EcoR*I restriction sites on both ends (5'-tgtgtaatacgactcactataggagggttactgatgagtccgtg-3' and 5'- tttttgttttcgtcctcacg gactcatcagtaaccct-3' for R1; 5'-tgtaatacgactcactataggtgaaaaactgatgagtccgtg-3' and 5'-ttccagttttcgtcctcaaggactcatcagtttttca-3' for Rm). The oligonucleotides were annealed and cloned to pcDNA3 (Invitrogen, San Diego, CA). The sequence and orientation of the ribozyme in the vector were confirmed by DNA sequencing.

DOTAP-mediated transfection of MCF-7 cells was performed according to the manufacturer's instructions. Briefly, cells seeded at a density yielding 50% confluency, were transfected with 5ug of pcDNA·Rm or pcDNA·R1 plasmid with 30ug of DOTAP. Six hours after transfection, the culture medium was replaced by selection medium containing G418 with a final concentration of 1.5mg per ml. The transfected cells were exposed to G418 for 1 month.

C. Characterization of transfectants

(A). RT-PCR analysis of ribozyme and hTR expression

Total RNA was isolated from the transfectants and the parental cells, and was reverse-transcribed by First Strand cDNA Synthesis kit (MBI, Hanover, MD) according to the manufacturer's instructions. To analyze ribozyme expression, the cDNA was amplified by using T7 and antisense primers complemented to 3' end of ribozyme (5'-tttttgtttcgtcc-3' for R1; 5'-ttccagtttagtcc-3' for Rm) and by performing 30 cycles of PCR (94°C for 30s , 48°C for 30s and 72°C for 30s). For the analysis of hTR and ß-actin expression, PCR amplification of 25 cycles was performed at 94°C for 30s, 59°C for 30s, and 72°C for 30s using the primers (5'-

gctctagagggttgcggagggtgggcct-3' and 5'-ctgcagtccccgggaggggcgaa-3' for hTR, 5'-caggtcatcaccattggcaatgagc-3' and 5'-cggatgtccacgtcacacttcatga-3' for ß -actin).

(B). Telomerase activity analysis

Telomerase activity was measured by TRAP assay as above described in Materials and Methods D.

(C). Population doubling

To evaluate the proliferation rate of transfactants, cells were seeded at 6well dish with $2x10^4$ densities per well. After culture for 1 week, cell numbers were counted by trypan blue staining. The population doubling (PD) / day was calculated by the following formula; $PD/day=\log_2$ (total cell number-$2x10^4$) / 7day

III. RESULTS

1. The minimal requirement of hTR for telomeric DNA binding was 326nt.

As shown in **Fig. 3 A**, the secondary structure of hTR is composed of template sequences and four conserved structural domains, namely, pseudoknot, CR4-CR5, Box H/ACA, and the CR7. This remarkable conservation of hTR structure implies an important role in telomerase function. To identify the significant structural domain of hTR in telomerase activation, we generated seven hTR truncation constructs based on the secondary structure. Truncations of hTR were designed to delete each structural domain in a stepwise way as shown a line diagram in **Fig. 3 B**. The size and integrity of *in vitro* transcribed hTR transcripts were confirmed by agarose electrophoresis with size marker (**Figure 3 C**).

We first identified telomere binding region of hTR by EMSA using *in vitro* transcribed hTR and the single strand telomeric repeated sequence, (TTAGGG)n. The optimum temperature for the telomeric DNA–hTR complex was found to be 37℃ compared with the other temperatures examined (24℃, 4℃, and 0℃; data not shown). To observe the effect of telomeric length on hTR-telomeric DNA complex formation, variable length

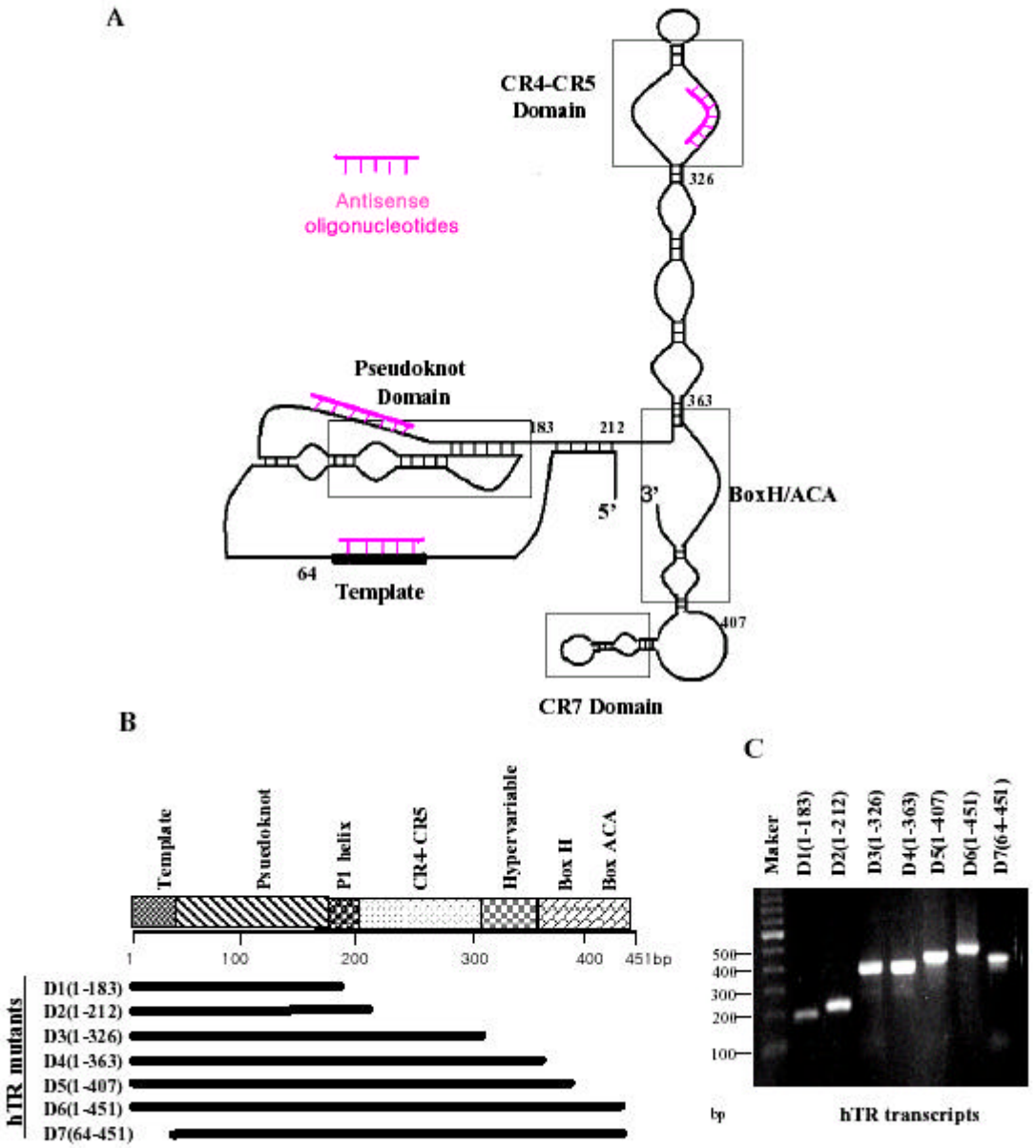

Figure 3. Secondary structure of hTR and schematic representation of hTR mutants. A. Secondary structure of hTR is composed of telomeric template sequence and several conserved domains including pseudoknot, CR4-CR5, Box H/ACA, and the CR7 domains (in box). Red regions showed the location of antisense oligonucleotides against template, pseudoknot, and CR4-CR5 domain, respectively. B. hTR mutant constructs are represented schematically with respect to the conserved structural domains. hTR mutants are deigned to serially truncate structural domains from 3'or 5'end of hTR and denoted by the transcribed nucleotides within hTR. C. hTR mutants are *in vitro* transcribed with T7 RNA polymerase and electrophoresed on 1.5% agarose-2.2M formaldehyde gel to verify the accurate sizes and amounts.

of (TTAGGG)n were added to the reaction (**Figure 4 A**). The strongest complex formation was observed only with $(TTAGGG)_3$ (lane 2). Shorter (lane1) or longer (lanes3, 4, and 5) telomeric sequences than 18mer did not form a sufficient complex compared to 18mer, $(TTAGGG)_3$. This result suggests that the length of the telomeric sequence was found to be critical for binding between hTR and telomeric DNA. Next, We identified the minimal structural domains of hTR required for telomere binding utilizing EMSA with hTR mutants and $(TTAGGG)_3$ (**Figure 4 B, top**). A binding complex was not observed in D1 (1-183), D2 (1-212) and D7 (64-451). The RNA-DNA complex formation increased gradually from D3 (1-326) to D6 (1-451). Mutant D3 (1-326), D4 (1-363) and D5 (1-407) showed 13.2%, 27.4% and 14.6% of the telomeric DNA binding activity comparing to the wild type D6, respectively. Thus, these EMSA results suggested that the minimal requirement of hTR for telomeric DNA binding was 326nt fragment, which contained the template, pseudoknot, and the CR4-5 domain. To monitor the amount and integrity of input hTR mutants in binding assay, we isolated RNA from binding mixture and performed northern hybridization with hTR-specific probe (**Figure 2B, bottom**). We observed that the levels and integrities of hTR mutants in binding assay were not grossly different. In spite of equal RNA amount, mutant D5 showed more reduced complex formation than the

shorter mutant, D4. Thus, we tested D5 as a putative dominant negative mutant in the next experiment.

2. Telomerase reconstitution activity of hTR mutants correlated with the telomere binding capacity.

To analyze the ability of each hTR mutant to reconstitute telomerase activity, we performed *in vitro* telomerase reconstitutional assay in an RRL system expressing hTERT and verified telomerase activity by TRAP assay. To establish the optimal reconstitution reaction, we examined the dose-response relationships of the recombinant components. Telomerase activity was shown to increase gradually by adding amount of two essential components of telomerase, hTR or hTERT (**Figure 5 A**). Active telomerase assembly was also found to be dependent on the presence of fresh RRL in a dose-dependent manner. Thus, we optimized the *in vitro* telomerase reconstitution reaction by adding 150 ng of hTR transcript, 2 μL of rabbit reticulocyte lysate expressing hTERT, and 2 μL of fresh rabbit reticulocyte lysate. The different hTR mutants were introduced into *in vitro* synthesized hTERT in the presence of fresh RRL (**Figure 5 B**). The activity of the reconstituted telomerase was measured using the TRAP assay. The results showed that D1 (1-183), D2 (1-212) and D7 (64-451) had no activity. D3 (1-

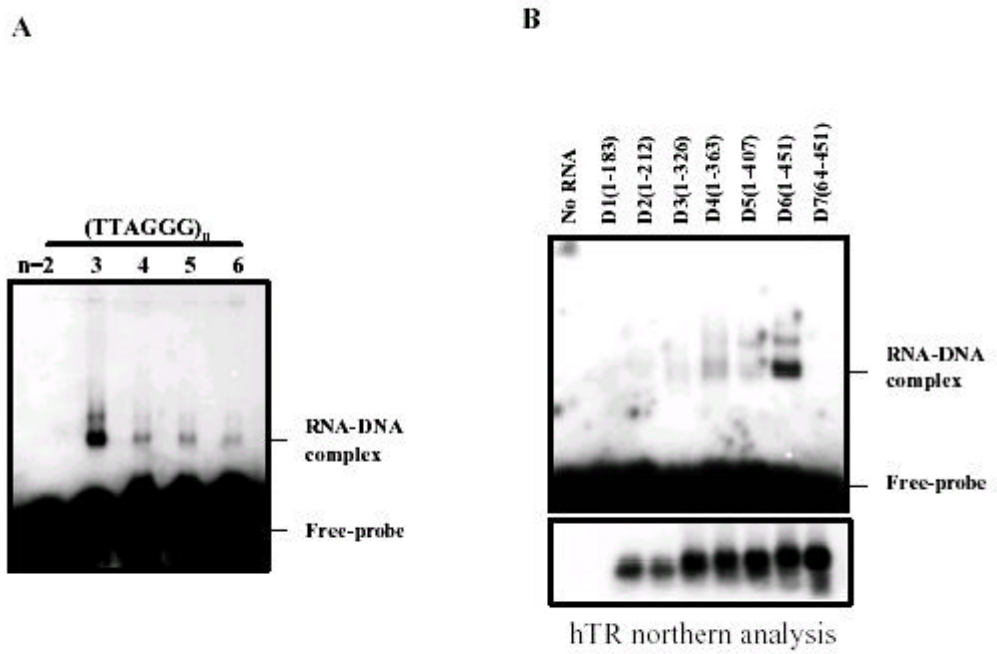

Figure 4. Complex formation between *in vitro* transcribed hTR and telomeric DNA. A. Effect of telomeric DNA length on DNA-RNA complex formation was evaluated by using different lengths of ^{32}P-labeled (TTAGGG)n (n=2, lane1; n=3, lane 2; n=4, lane 3; n=5, lane 4; n=6, lane 5). B. EMSA was carried out using ^{32}P-labeled telomeric DNA, $(TTAGGG)_3$ and various truncated hTR mutants transcribed *in vitro.* One pmole of ^{32}P-labeled probe was incubated without (lane 1) or with (lane 2-8) the truncated hTR mutants. Reactions were resolved in 1.5 % agarose gel and visualized by autoradiography (top). After binding reaction, isolated hTR mutants were subjected the northern hybridization analysis with ^{32}P-labeled oligonucleotides complementary to hTR nt+164 to nt+183 (bottom). .

326), D4 (1-363) and D5 (1-407) showed 46%, 58.0 %, and 32.0% of the telomerase activity comparing to the wild type D6, respectively (**Figure 5 B**). The results showed that at least, 326nt of hTR is essential for telomerase activity in RRL reconstitution system, consistent with the previous studies [34, 37]. We also found that telomerase activity correlated with the telomere binding capacity in each mutant, implying that the minimal hTR length contributes to both telomeric DNA binding and telomerase enzymatic activity. However, there were some discrepancies in degree of both abilities. For example, mutant D3 expressed 46 % of telomerase activity of wild type hTR, but is only exhibited 13.2 % of telomere binding ability of wild type hTR. To exclude the experimental bias from single concentration point of hTR, we tested three different concentrations of hTR mutant in binding assay (data not shown) and telomerase reconstitution assay (**Figure 5 B**). Increment of hTR concentration did not change activities in both assays, suggesting that ability of hTR to make few complexes with telomeric DNA sufficient for confer the telomerase activity. This finding was also observed in functional study using antisense oligonucleotides (**Figure 5 A and B**). Regardless of complete inhibition of telomere binding by anti-template oligonucleotide, telomerase activity still exhibited 29.6 % of telomerase activity of wild type hTR.

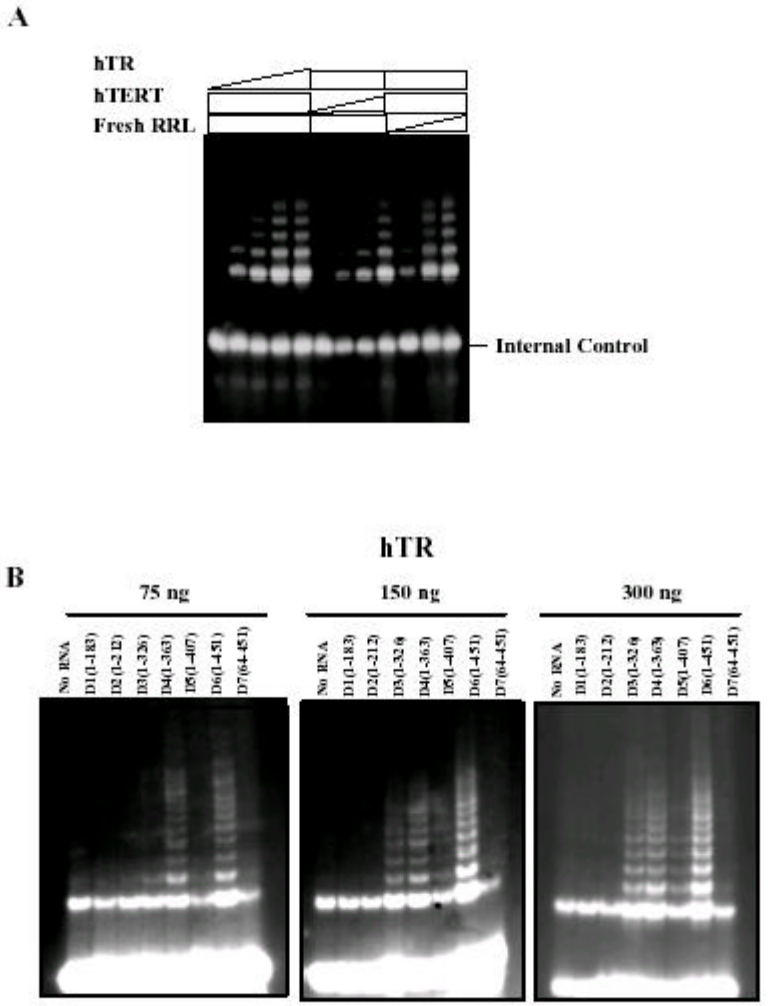

Figure 5. ***In vitro*** **reconstitution of telomerase activity. A. Standard assay was performed with 150 ng hTR, 2 µL of** ***in vitro*** **synthesized hTERT and 2 µL of fresh RRL.** ***In vitro*** **reconstitution of telomerase was evaluated with various amounts of hTR, hTERT, and fresh RRL (lanes 1-5; 0, 10, 50, 150, or 300 ng of hTR, lanes 6-9; 0, 0.5, 1, or 2 µL of hTERT synthesized** ***in vitro,*** **lanes 10-12; 0, 1, or 2 µL of fresh RRL). B.** ***in vitro*** **reconstitution assay was performed with various amounts of** ***in vitro*** **transcribed hTR (left; 75ng, middle; 150 ng; right; 300 ng). The telomerase activity of** ***in vitro*** **reconstitution was measured by TRAP assay.**

3. Structural domains of hTR played functionally a distinct role in telomere binding and hTERT binding.

To evaluate the ability of each hTR mutant to associate with hTERT, assembly reaction mixtures were immunoprecipited with HA-antibody to HA tag located at the carboxy terminus of hTERT, and subjected to western and northern analysis (**Figure 6 C**). hTERT binding activity of each hTR mutant was compared to the telomerase enzymatic activity of HA-immunoprecipitates (**Figure 6 A**). Total hTERT level in assembly reaction mixture and pulled down precipitates with HA antibody were same in all mutants (**Figure 6 B**). In spite of the same input amount in all assembly reaction fractions, mutant D7 spanning nucleotides 64-451 did not coimmunoprecipitate with hTERT. These results suggest that 1-64nt structural region of hTR plays a critical role for binding with catalytic subunit of telomerase and mutant D1 spanning 1-183 is sufficient for interaction with hTERT. Furthermore, it suggests that minimal structural domains of hTR play functionally a distinct role in telomere binding and hTERT binding.

We summarized the relative ratio of the enzymatic activity, telomeric DNA binding and hTERT binding of each hTR mutant in **Table 2**. There was a difference of structural length of hTR required for hTERT and telomere binding, respectively. On the contrary, minimal sequences of hTR required for

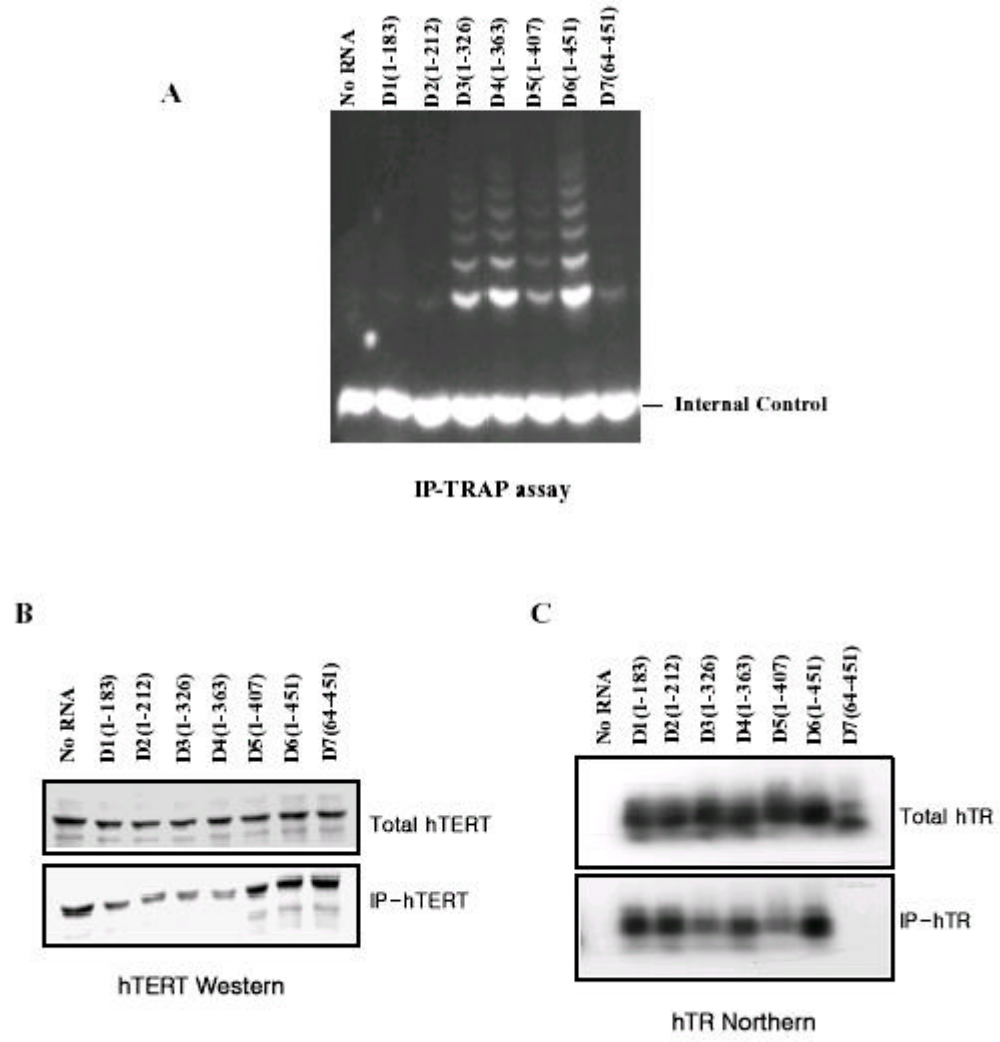

Figure 6. Telomerase enzymatic activities and hTERT binding abilities of hTR mutants. Different mutants were reconstituted with hTERT containing a HA tag, and were subjected to an anti-HA immunoprecipitation. A. Telomerase activity analysis of the anti-HA immunoprecipitants by TRAP assay. B. Before (top) or after (bottom) the immunoprecipitation, western analysis was performed with anti-HA monoclonal antibody. C. Before (top) or after (bottom) immunoprecipitation, isolated RNA was subjected to northern hybridization analysis with hTR-specific probe.

telomerase activity and the telomere binding were exactly coincided. These findings demonstrated that telomere binding of hTR directly influences the reconstitution of telomerase activity. Hence, in a sense of determining the minimal requirement for telomerase activity reconstitution, hTR-telomere binding is a limiting factor rather than hTR-hTERT binding.

4. Targeted disruption of the template and CR4-CR5 region resulted in efficient inhibition of the hTR-telomeric binding and telomerase activity.

As shown in **Fig. 2** and **3**, the minimal sequence of hTR required for telomere binding and telomerase activity contains three functional domains, i.e., the template, pseudoknot, and CR4-CR5 domains. To evaluate the role of the each functional domains in the minimal hTR sequences, we used antisense oligonucleotides targeting each domain. Furthermore, these antisense oligonucleotides were tested to determine any possible role as a telomerase inhibitor. Antisense oligonucleotides were designed to target the unpaired region in template, pseudoknot, or CR4-CR5 domain of hTR based on the proposed secondary structure (**Figure 3 A**). **Figure 7 A** shows that wild-type hTR forms a strong complex with the ^{32}P-labeled telomeric sequences of $(TTAGGG)_3$ (lane 1). This RNA-DNA complex formation was competitively inhibite by adding a non-labeled homologous competitor (lane 2), whereas it

Table 2. Summary of hTERT biding, telomeric DNA binding, and telomerase activity of hTR truncations.

Telomerase RNA	Characterazation of structure	hTERT Binding	Telomeric DNA Binding	Telomerase Activity
hTR D1(1-183)	Truncate 3'terminal 268nt; partly disrupt P1 helix, delete CR4-CR5 domain, BoxH/ACA and CR7domain	+++++	–	–
hTR D2(1-212)	Truncate 3'terminal 239nt; delete BoxH/ACA and CR7domain	+++++	–	–
hTR D3(1-326)	Truncate 3'terminal 125nt; partly disrupthypervariable region, delete BoxH/ACA and CR7domain	++++	+	+++
hTR D4(1-363)	Truncate 3'terminal 88nt;delete BoxH/ACA and CR7domain	+++++	++	+++
hTR D5(1-407)	Truncate 3'terminal 44nt;partial distrupte BoxH/ACA , delete CR7domain	++++	+	++
hTR D6(1-451)	Full length, wild type	+++++	+++++	+++++
hTR D7(64-451)	Truncate 5'terminal 64nt;delete template region	-	-	-

¶ Relative activity was determined by defining the relative level of activity taking wild type hTR as 100%. The amount of activity for all mutants represented symbolically as a relative range: +++++; greater than 80%, ++++; between 79%-60%, +++; between 59%-40%, ++; between 39%-20%, +; between 19%-1%, -; less than 1%.

was not inhibited by any of the equal molecular weight, nonspecific primers [$(TGTGAG)_3$, lane 3; and $(TTGGAG)_3$, lane 4]. This finding suggested that the formation of the hTR-telomere complex is mediated by a sequence-specific recognition. Anti- template oligonucleotides completely abolished the retarded RNA-DNA band (lane 5), while anti-CR4-CR5 oligonucleotides reduced only 4% (lane 7). On the other hand, anti-pseudoknot oligonucleotide enhanced the complex formation (lane 6). These results suggested that the template region is a good target site for the inhibition of telomere binding.

We analyzed the effects of these antisense oligonucleotides on telomerase activity by TRAP assay (**Figure 7 B**). Anti-CR4-CR5 and anti-template oligonucleotides effectively inhibited telomerase activity by 90.6% and 70.4%, respectively. Anti-pseudoknot oligonucleotides also reduced telomerase activity by 25.9% compared with that of the wild type. Since the antisense oligonucleotides showed different effects in the EMSA and the TRAP assay, these findings demonstrate that each structural domain play distinct roles in telomere binding and telomerase activity.

5. D5 is a putative dominant negative mutant of hTR.

As shown in **Fig. 4 A** and **6 A**, mutant D5 reduced telomere binding and telomerase activity than the shorter mutant, D4. We assumed D5 to be a

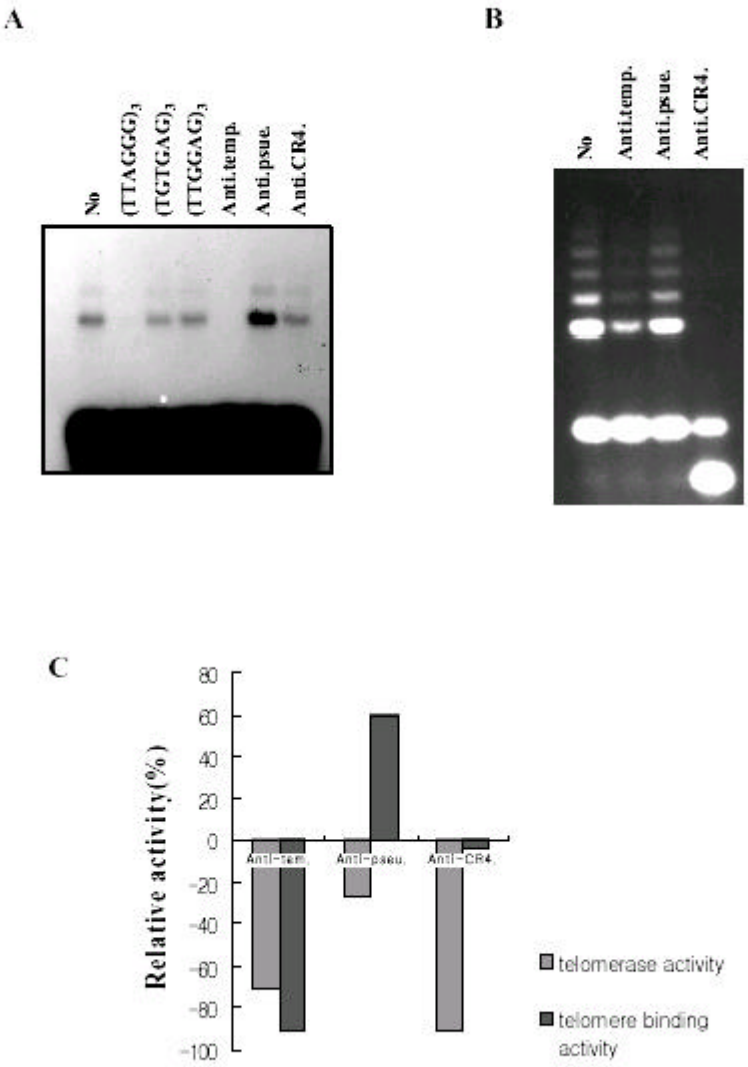

Figure 7. Effect of antisense oligonucleotides on the formation of RNA-DNA complex and telomerase activity. A. EMSA was performed in the absence (lane 1) or presence (lanes 2-7) of competitive primers or antisense oligonucleotides. hTR was incubated with non-labeled homologous competitor (lane 2), nonspecific primer [$(TGTGAG)_3$, lane3: $(TTGGAG)_3$, lane4], anti-template oligonucleotide (lane 5), antipseudoknot oligonucleotide (lane 6), or anti-CR4-CR5 oligonucleotide (lane 7) prior to adding the ^{32}P-labeled probe. B. After incubating hTR with antisense oligonucleotides, hTERT was added to the reaction and the TRAP assay was performed. C. relative quantitation of the degree of inhibition by antisense oligonucleotides. .

dominant negative mutant against wild type hTR. The addition of D5 to wild type hTR resulted in a gradual decrease of telomerase activity, in a dose-dependent manner (**Figure 8 A**). Telomerase activity of the wild type was also completely inhibited by adding the equal amount of D5. As shown in **Fig. 2 and 3**, the alteration of the hTR structure in D5 inhibited the assembly of wild type telomerase significantly. These results suggested D5's role as a putative dominant negative mutant, which inhibits wild type hTR.

6. Specific cleavage of a synthetic RNA substrate by the R1 ribozyme

Seven kinds of hammerhead ribozyme were designed to target the NUX sequences (where N can be any ribonucleotides, and X can be any ribonucleotides excepting guanine) in human telomerase RNA, base on the open loops in the proposed secondary structure. First, we studied whether the seven kinds of ribozyme could cleavage the RNA substrate efficiently in cell-free system. A ^{32}P-labeled hTR RNA substrate of 451bp was mixed with each ribozyme at a molar ratio of 1:5, and incubated in cleavage reaction buffer at 28 ℃ for 16hrs. As shown in **Fig. 9**, reaction without ribozyme and reaction with a mis-matched ribozyme did not show any cleaved fragments. Ribozyme R1 efficiently cleaved the 451-bp telomerase RNA substrate into 362 and 45 bp fragments. The ribozyme R2, 6, and 7 showed cleavage activity, while

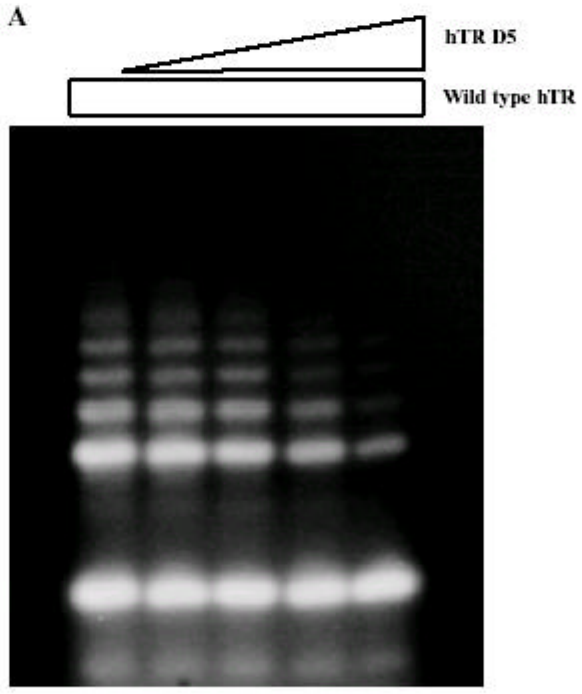

Figure 8. Inhibition of wild type hTR by D5 mutant. Inhibition of wild type hTR by mutant D5 was assayed by TRAP assay (A). Wild type hTR (150 ng) was incubated with 0, 18.7, 37.5, 75, or 150 ng of D5 mutant prior to adding RRL expressing hTERT. The reconstituted telomerase was subjected to TRAP assay. .

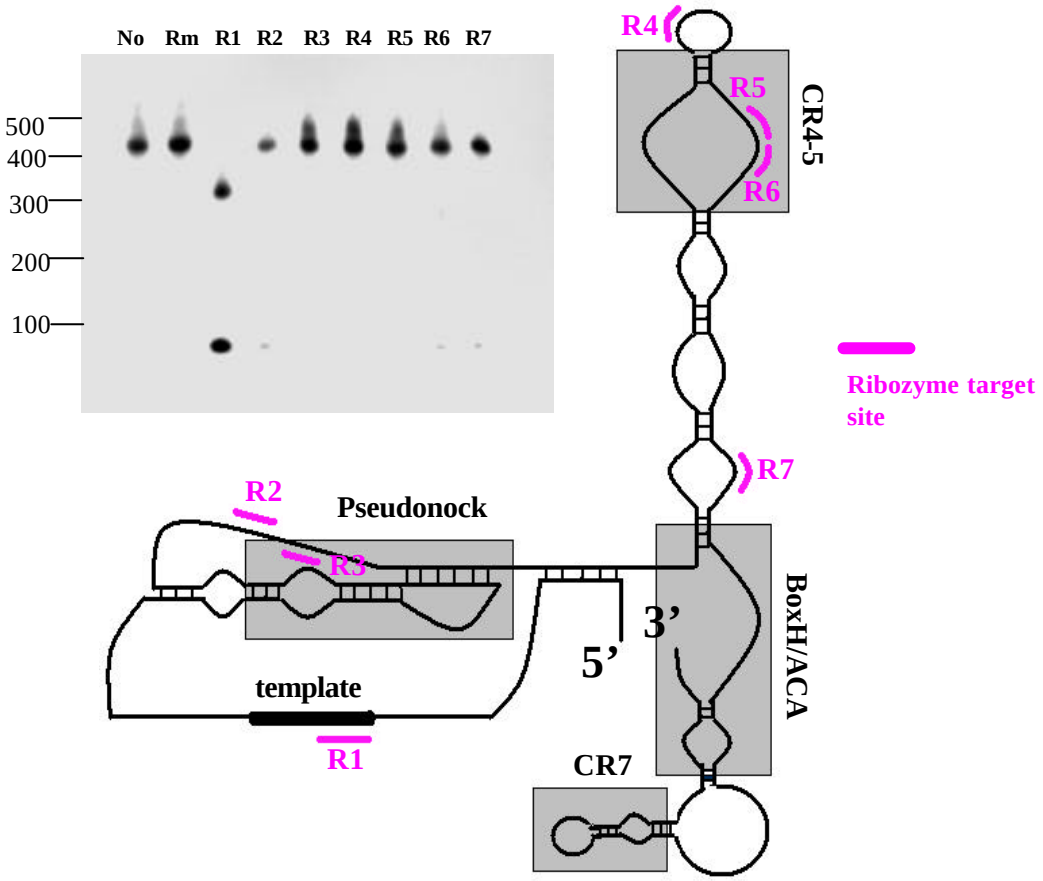

Figure 9. Target sites of ribozymes against hTR and *in vitro* cleavage assay. Hammerhead ribozyme were designed against seven NUX sequences in open loops on hTR secondary structure and represents in red. *In vitro* cleavage assay was performed with a $^{-32}$P UTP labeled hTR substrate and ribozymes targeted to different sites of hTR. The reaction mixture was eletrophoresed in 6% polyacrylamide-7M urea gel.

ribozyme R3, R4, and R5 did not show any RNA substrates cleavage effect.

7. Inhibition of telomerase activity by the exogenously delivered - ribozyme R1

For the screening of potency of the ribozymes, the ribozymes were synthesized exogenously and delivered using cationic loposome. The ribozymes were introduced to human breast tumor cell line MCF-7 with DOTAP. At 72hrs after administration of ribozyme, telomerase activity was determined by TRAP assay of cell lysates. Telomerase activity was diminished when ribozyme R1 was introduced (**Figure 10**). DOTAP alone or mis-matched ribozyme did not induce any effect on telomerase activity. None of the other ribozymes showed any significant change of telomerase activity comparing to that of control cell. It was thus concluded that ribozyme R1 was the most promising in the transfection study.

8. Degradation of hTR by stable expression of ribozyme R1 attenuated telomerase activity

We subcloned the ribozyme R1 or Rm sequences into pcDNA3 vector and introduced it into MCF-7. The transfectants were selected by selection medium containing G418 to a final concentration of 1.5mg per ml for 1month.

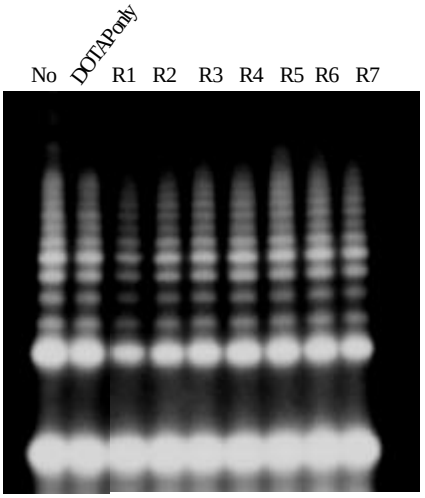

Figure 10. Inhibition of telomerase activity by exogenous delivery of ribozyme R1 RNA into human breast tumor cell line MCF-7. Five microgram of *in vitro* transcribed ribozyme was transfected with DOTAP into MCF-7 cell, and incubated for 72 hours. Ribonucleoprotein was extracted from transfectants and analyzed telomerase activity by TRAP assay.

After 1 month of selection, 8 Rm clones and 16 R1 clones were obtained from transfectants. To evaluate the expression of ribozyme and hTR from each clone, RT-PCR was performed with hTR, and ribozyme-specific primers (**Figure 11**). We confirmed that all clones of MCF-7 stably expressed the ribozyme either R1 or Rm. Rm-expressing clones did not show any change in hTR expression compared with that of parental cells. On the contrary, hTR expression was significantly reduced in clones which expressed ribozyme R1 suggesting that ribozyme R1 successfully degraded hTR RNA. In some clones (clone 4, 5, 6, 9, and 12), hTR expression was detectable feebly. Telomerase activity of each transfectants was also measured by TRAP assay (**Figure 12**). Telomerase activity of R1 transfectant was dramatically attenuated compared with parent or Rm transfectants, although the activity level differed in the clones. Clone 1, 2, 3, 7, 8, 12, and 14 showed the undetectable level of telomerase activity, while clone 6, 9, and 15 still expressed a weak telomerase activity. Telomerase activity in transfectants correlated well with the remaining hTR expression level (**Figure 12, bottom**) suggesting that specific degradation of hTR by ribozyme R1 resulted in the inhibition of telomerase activity.

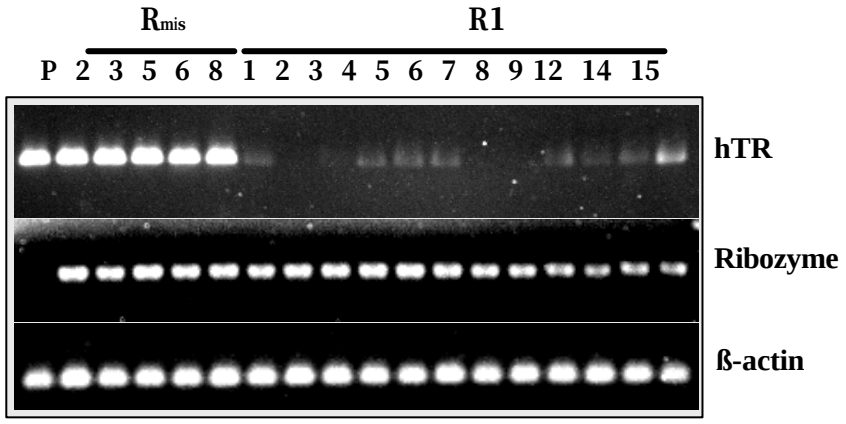

Figure 11. Specific degradation of hTR by stable expression of ribozyme R1. Ribozyme Rm or R1 expression vector was transfected into MCF-7 cell. Clones were selected with selection media containing G418 for 1 month. Total RNA was isolated from each clone and performed RT-PCR with b-actin, ribozyme, and hTR-specific primers.

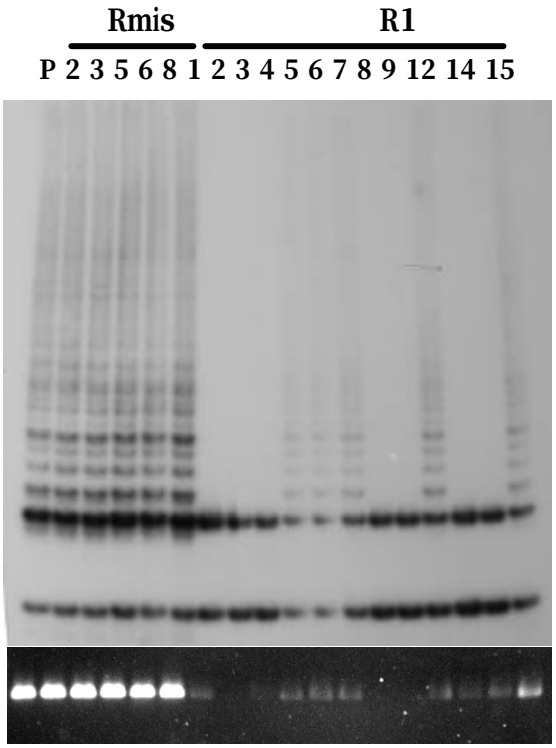

Figure 12. Degradation of hTR by stable expression of ribozyme R1 inhibited telomerase activity in human breast tumor cell line MCF-7. Ribonucleoprotein was extracted from transfectants and analyzed telomerase activity by TRAP assay. Low panel showed hTR expression of transfectants.

9. Clones with attenuated telomerase activity showed reduction of replicative capacity.

To evaluate anti-proliferative effect of ribozyme R1, we measured population doubling (PD)/day. In **Fig. 13 A**, parental cells proliferated at the rate of 0.69 PD/day, and Rm transfectant showed 0.68 PD/day similar to that of parent cells. On the contrary, R1 transfectants showed a significantly delayed growth rate of 0.48 PD/day. Especially, clone 2 and 7 showed a half of the proliferation rate of parental cells, whereas clone 15 steadily proliferated at the level of the parental cells. Replicative capacity of transfactants paralleled with the level of telomerase activity (**Figure 13 B**). The results suggested that attenuation of telomerase activity by stable R1 ribozyme expression resulted in growth inhibition of MCF-7 cell line.

A. Growth rate of transfectants

Ribozyme	Clone	PD/day	Mean (±SD)
—	P	0.69	**0.69**
Rm	C2	0.68	**0.68 (± 0.032)**
	C3	0.71	
	C5	0.64	
	C6	0.65	
	C8	0.71	
R1	C1	0.46	**0.48 (± 0.078)**
	C2	0.37	
	C3	0.43	
	C4	0.44	
	C5	0.48	
	C6	0.58	
	C7	0.38	
	C8	0.52	
	C9	0.51	
	C12	0.47	
	C14	0.46	
	C15	0.65	

B.

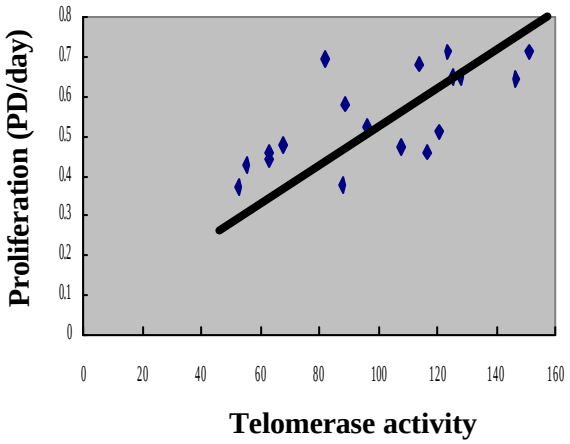

Figure 13. Anti-proliferative effect of ribozyme R1 and relationship between telomerase activity and cell proliferation rate. After 2 months of transfection, cells were seeded in 6 well dish and cultured for 1 week, and then cell number was counted. The population doubling (PD)/day was calculated by the formula, PD/day=$\log_2$ (total cell number-$2x10^4$)/7day (A). Proliferation rate of each transfectant was compared with its telomerase activity (B).

IV. DISCUSSION

Telomerase accessibility to telomere is essential for the synthesis of new telomeric sequences. Since hTR carries the template sequence complementary to telomeres, hTR would appear to be the most responsible subunit among the telomerase components in terms of access to telomeres. To explore the direct interaction between hTR and telemeres, we developed an EMSA using *in vitro* transcribed hTR and ^{32}P-labeled telomeric-repeated sequences. The length of the telomeric sequence was found to be critical for binding between hTR and telomeric DNA. Shorter sequences than 18mer were too short to make a stable base pair with hTR, whereas longer sequence than 18mer would form secondary structures themselves, e.g., the G-quartet [42, 43].

Previous studies using different telomerase reconstitution systems discovered roles of the functional domain of hTR in both telomerase activity and interactions with other telomerase components. Narayanari *et al.* reported that nucleotides +44-+203, which contains the template and pseudoknot domains, is the minimal sequences required for telomerase activity in a telomerase reconstitution assay using MNase treated human cell extract [35]. Another group also suggested that nucleotides +33-+325 of hTR, which contains the template, pseudoknot and CR4-CR5 domains were the minimal

requirement for telomerase activity in an RRL system expressing hTERT [37]. In particular, the integrity of CR4-CR5 and the pseudoknot structure were found to be critical for the reconstitution of telomerase activity in studies using truncated or sequence-substituted hTR mutants [39, 40]. Direct interaction of hTR-hTERT was also investigated by using an *in vitro* telomerase reconstitution assay. Autexier *et al*. reported that 159nt of hTR was sufficient for a stable interaction with hTERT and found that two distinct hTERT-binding sites exist at nucleotides + 33- +147 which contains the template region and a part of the pseudoknot domain, and at +164- +325 which contains the pseudoknot domain in part and the CR4-CR5 domain of hTR [38]. Interestingly, different structural length of hTR is required for hTERT binding and telomerase activity, and the telomerase enzymatic activity needs much longer fragment of hTR than hTERT binding, which implied that there are other regulatory events excepting hTERT binding to confer telomerase enzyme activity. Thus, we focused on interaction between hTR and the telomeric DNA. Results showed that the minimal region of hTR required for telomere binding is up to 326nt, which contains the template, pseudoknot and CR4-5 domain. We also confirmed that this 326nt of hTR is critical for the assembly of telomerase activity. These findings demonstrated that telomere binding of hTR directly influences the reconstitution of telomerase activity.

Hence, in terms of determining the minimal requirements for the expression of telomerase activity, the present study showed that hTR-telomere binding is a limiting factor rather than hTR-hTERT binding.

Many experiments have been conducted with a view to inhibit telomerase in cell culture system and in animal models. Application of reverse transcript inhibitors such as azidothymidine (AZT) and dideoxyguanosine (ddG) was found to lead to telomere shortening [15, 16]. Telomerase-targeting antisense oligonucleotide induced apoptosis, differentiation of cancer cells and reduced tumorigenicity [17]. We evaluated the role of each domain of hTR in telomere binding and in the reconstitution of telomerase activity with antisense oligonucleotides. Furthermore, these antisense oligonucleotides were tested to determine any possible role as telomerase inhibitors. Antisense oligonucleotides were designed to target the unpaired region in template, pseudoknot, or CR4-CR5 domain of hTR based on the proposed secondary structure by Chen *et al.* [33]. Previous efforts have demonstrated that conserved domains of hTR played a distinct role in telomerase activation. For example, the first, sequence 5'-CUAACCCU-3' in the template region of hTR provided a template sequence for the synthesis of new telomeric repeats [44]. In this study, we demonstrate that the template region plays an important role in telomerase reconstitution not only by serving as a template, but also by

binding to the telomeric DNA. The pseudoknot domain consisted of three helix structures that stabilize the secondary structure [45]. This domain was reported to be required for telomerase activation and assembly [39]. The present study showed that the disruption of the pseudoknot domain by antisense oligonucleotide reduced telomerase activity, but in contrast, it enhanced telomere binding of hTR. These findings suggest that the pseudoknot domain might contribute to binding with hTERT, and that this domain is essential for telomerase activity, whereas it did not grossly influence the binding activity to telomeres. Antisense oligonucleotide targeting the pseudoknot domain might stabilize the secondary structure of hTR resulting in the enhancement of stable binding with telomeres.

The CR4-CR5 domain lies downstream of the pseudoknot domain, and is composed of a stem-loop structure. The roles of the CR4-CR5 domains have not been demonstrated clearly, but truncated mutant without CR4-CR5 domain did not have telomerase activity [40]. CR4-CR5 domain has an open long single strand in its loop structure. Therefore we considered this region as a good target site for telomerase inhibition. We found that the antisense oligonucleotide targeting CR4-CR5 domain partially inhibited telomeric DNA-hTR binding, but completely inhibited its enzymatic activity. It was also reported that telomerase activity can be reconstituted in the presence of two

inactive, nonoverlapping fragments of hTR [38]. These fragments are composed of the pseudoknot and the CR4-5 domains. In our study, antisense oligonucleotides targeting the pseudoknot and the CR4-5 domains inhibited the reconstitution of telomerase activity, whereas they did not influence telomere binding ability. This finding suggested that two functional domains (pseudoknot, and CR4-CR5 domains) are more critical for binding with hTERT than binding with telomeres.

As shown in **Fig. 4 and 6**, the alteration of the hTR structure in D5 inhibited the assembly of wild type telomerase effectively. These results suggested D5's role as a putative dominant negative mutant, which inhibits wild type hTR. Although it had more 44nt at the 3'-terminus than D4 (1-363), D5 showed less telomere binding ability and enzymatic activity than D4. At this point, more detailed function study of D5 remains to be done, but at least, it can be suggested that D5 behaves as a dominant negative mutant though dimerization or polymerization with native hTR. Lingner *et al.* showed that telomerase RNA is assembled into active telomerase as a dimeric structure [46]. Therefore, a telomerase heterodimer composed of wild type and mutant telomerase RNA might be inactive compared with the wild type homodimer. The 363-407 sequences of D5 remained as a single stranded overhang, which does not form a natural secondary structure. The single stranded overhang

might affect wild type hTR by aberrant base pairing with hTR, which this induce dysfunction of wild type hTR. Currently, we are investigating whether D5 can inhibit wild type hTR in cancer cells.

Specific inhibition of telomerase in tumor cells led to telomere shortening, and eventually, to cell death [15, 16, 17, 18, 19]. Here, we studied anti-telomerase effect of hammerhead ribozyme targeting telomerase RNA component. Telomerase activity is not associated with the expression level of telomerase RNA in some tumors and cells. However, the crucial role of the RNA component in telomere shortening has been demonstrated in telomerase RNA knock mouse [47]. Thus, we reasoned in this experiments that the breakdown of telomerase RNA by the ribozymes must lead to the attenuation of telomerase activity. because telomerase RNA is a direct participant in the telomerase complex as an essential element. We demonstrated that the reduction of telomerase RNA expression was associated with the attenuation of telomerase activity.

We chose the 7 NUX sequences in telomerase RNA including template region. Six target sites of telomerase RNA excepting template region were not cleaved efficiently by hammerhead ribozyme. For the ribozymes to recognize and catalyze the target sites, the target region must be sufficiently exposed on the outer surface of the molecule. Based on the data that ribozyme R1

targeting the template region reduced the telomerase activity most efficiently, the template region must be located at the outer surface of telomerase molecule.

V. CONCLUSION

We first demonstrated 326 nucleotides of hTR spanning the template, pseudoknot, and CR4-CR5 domains exhibited a binding capacity with telomere and a telomerase activity *in vitro* reconstitution assay. This finding suggested that telomere binding ability is critical for telomerase enzymatic activity. Furthermore, targeted disruption of the template region of hTR resulted in the complete blocking of the hTR-telomeric binding, whereas disruption of CR4-CR5 totally abrogated telomerase activity *in vitro*. It is suggest that each functional region may underlie in different regulation in a sense of telomere binding and telomerase activity. In ribozyme study, the template region of hTER was a good target for inhibition of telomerase activity, as anticancer strategy using ribozyme in human breast tumor cell.

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국문요약

사람의 telomerase RNA가 telomere와 결합하는 부분과 이를 표적으로 하는 라이보자임의 효과 검정

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Telemerase는 염색체 말단에 있는 telomere를 연장, 유지시켜주는 역전사 효소이다. 사람의 telemere는 TTAGGG서열이 반복된 구조로서 염색체 기능과 구조 유지에 필수적인 역할을 한다. 이러한 telemere는 세포가 분열할 때 마다, DNA polymerase가 가지는 'end replication problem'에 의해 점점 짧아진다. telomere가 일정길이 이상 짧아진 세포는 더 이상 분열하지 못하고 노화나 세포사멸 기전에 의해 제거되어진다. 하지만 정상 체세포와 달리 암세포에서는 telomerase가 과발현됨으로서 끊임없이 성장, 분열하는 불멸화 능력을 획득한다. 따라서 암치료에 있어 telomerase는 효과적이고 암세포 특이적인 표적분자로 제시된다.

Telomerase는 크게 두개의 구성요소로 이루어져 있다. 하나는 역전사 효소 기능을 가지는 단백질 부분인 hTERT와 telemere의 주형으로 역할을 하는 RNA인 hTR로 이루어져 있다. hTR은 모든 척추동물에서 잘 보존된 이차 구조를 가지고 있으며 template, pseudoknot,

CR4-CR5, Box H/ACA, CR7와 같은 특징적인 구조로 이루어져 있다.

본 연구는 hTR이 telomere와 결합하는데 필요한 domain에 대해 알아보고자 하였고, 이것을 바탕으로 하여 이 domain을 억제할 수 있는 ribozyme을 합성하여 항암요법으로서의 가능성을 알아보고자 하였다. 먼저 hTR의 각 domain을 차례로 삭제한 truncation mutant를 제작하였다. 이러한 mutant들이 telomere와 결합하는 능력을 electrophoretic mobility shift assay (EMSA)로 시행하였다. 결과는 326nt를 가지고 있는 mutant에서부터 결합이 관찰되었다. 따라서 hTR이 telomere와 결합하는데 최소한 326nt가 필수적임을 알 수 있었다. 본 연구자는 이 326nt내에 포함된 있으며 template, pseudoknot, CR4-CR5 domain의 역할을 좀더 알아보고자 각각의 domain에 대한 antisense oligonucleotide를 제작하여 telomere와 결합하는 능력과 효소활성에 미치는 영향을 알아보았다. 결과는 template에 대한 antosense oligonucleotide는 telomere와의 결합과 효소활성을 모두 효과적으로 억제하였다. 반면 CR4-CR5에 대한 antisense oligonucleotide는 효소활성은 효과적으로 억제하였으나 telomere와 결합하는데는 큰 영향을 미치지 못하였다.

이러한 *in vitro* 결과를 토대로 하여, hTR을 표적으로 하는 7개의 ribozyme을 제작하였다. 7개의 ribozyme을 *in vitro*에서 합성한 hTR substrate와 반응 시킨 결과 R1 ribozyme에서 효과적으로 hTR이 잘려짐을 관찰하였다. 따라서 R1을 발현하는 expression vector를 제작하여 사람의 유방암 세포주인 MCF-7에 transfection하였다. 이 transfectant로 부터 hTR의 발현이 감소하고 이에 의해 telomerese 효소활성도가 감소되는 것을 관찰하였다. 긍극적으로 이러한 세포에서 R1 ribozyme에 의해 암세포의 성장이 억제됨을 확인하였다.

결론적으로 본 실험은 hTR이 telomere와 결합하는데 326nt가 필수적임을 확인하였다. Antisense oligonucleotide 실험을 토대로 각각의 domain은 telomerase가 활성화되는데 서로 다른 기능을 담당하고 있음을 확인하였다. hTR의 template domain을 표적으로 한 R1 라이보자임은 효과적으로 telomerese 활성과 암세포의 성장을 억제하였다.

따라서 hTR의 template domain은 암세포의 telomerase활성을 억제하는 좋은 target site임을 확인하였다

Key Words: Telomere; Telomerase; Human Telomerase Protein Component hTERT; Human Telomerase RNA Component hTR; Cancer; Ribozyme.