Different role of functional domains of hTR in DNA binding to telomere and telomerase reconstruction

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Abstract Even if template sequence of hTR played an essential role in telomere binding, a 326 nucleotide fragment of hTR containing template, pseudoknot, and CR4–5 domains is critical for both binding with telomeric DNA and reconstitution of telomerase activity. A functional study with antisense oligonucleotides suggested that targeted disruption of the template region efficiently abrogated both telomeric DNA binding and telomerase activity, whereas disruption of the CR4–5 region induced only loss of telomerase activity. hTR interacts with telomeric DNA via structural region composed of the template, pseudoknot, and CR4–5 domains, however, each structural domain plays a distinct role in telomere binding and telomerase activity reconstitution.
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1. Introduction

The synthesis of telomeric DNA requires at least two telomerase components: the protein catalytic subunit (hTERT) and the RNA subunit (hTR). A secondary structure of hTR based on phylogenetic comparative analysis shows that hTR contains several structural domains, which are conserved in all vertebrates [1]. The conserved secondary structure is composed of several distinct domains, which include the template, pseudoknot, CR4–5, Box H/ACA, and the CR7 domains.

Human telomerase activity can be reconstituted in three different in vitro systems, such as addition of in vitro transcribed hTR to rabbit reticulocyte lysates (RRLs) expressing hTERT [2], micrococcal nuclease (MNase, Pharmacia) treated cancer cell lysate [3], or recombinant hTERT purified from baculovirus-infected insect cells [4]. In vitro assembly reactions using deleted or site-directed hTR mutants suggest that the structural domains of hTR play critical roles in telomerase activation. Wright and co-workers [5] reported that a region between nucleotides +33 and +325 is required for telomerase enzymatic activity. It was also suggested that a 159 nucleotide fragment of hTR is sufficient for a stable interaction between hTR and hTERT [6]. The CR4–5 and pseudoknot domains

have also been suggested as critical domains for the activation of this enzyme [7,8]. However, the significance of the structural domains of hTR in the binding with telomeric DNA and the effects that this binding has on enzymatic activity have not been elucidated.

2. Materials and methods

2.1. Preparation of hTR

We generated truncated hTR mutants based on the secondary structure of hTR [2]. Full length or truncated mutants were obtained by PCR amplification of HT1080 genomic DNA with oligonucleotide primers encoding the appropriate hTR sequences: D1 (+1 and +183), D2 (+1 and +212), D3 (+1 and +326), D4 (+1 and +363), D5 (+1 and +407), D6 (+1 and +451), and D7 (+64 and +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).

2.2. Preparation of hTERT

Insert DNA containing the entire hTERT coding sequences fused with HA-epitope tag at C-terminal was subcloned into pCI neo vector at *Eco*RI–*Sal*I sites. hTERT-HA protein was synthesized by using a RRL transcription/translation system (TNT^R System; Promega). hTERT-HA cDNA was added to RRL at a concentration of 0.04 μg/μL and incubated at 30 °C for 90 min. The translation product was used for in vitro telomerase reconstitution.

2.3. In vitro telomerase reconstitution and TRAP assay

Human telomerase was reconstituted in a 5 μL 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 then incubated at 30 °C for 90 min and diluted 10-fold in Chaps lysis buffer (10 mM Tris–Cl, pH 7.5, 1 mM MgCl$_2$, 1 mM EGTA, 0.1 mM PMSF, 0.5% Chaps, and 10% glycerol). Telomerase activity was determined by using the TRAP assay as described by Kim and Wu [9]. The relative activity of the telomerase was determined using the following formula:

$$\text{TPG } (\%) = \frac{\text{Sample}[(T - B)/CT]}{\text{wild-type}[(T - B)/CT]} \times 100$$

where TPG is the telomerase activity, T the total intensity of telomerase mediated bands, B the intensity of the negative control, and CT the intensity of the internal control.

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

2.4. Electrophoretic mobility shift assay (EMSA)

EMSA was used to identify the telomere binding ability of the hTR mutants by using a ^{32}P-labeled telomeric sequence (TTAGGG)$_n$. In standard binding reactions, 150 μg of a hTR mutant was incubated

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with 1 pmol of end labeled (TTAGGG)$_3$ in 1× binding buffer (50 mM Tris–Cl, pH 7.5, 5 mM MgCl$_2$, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 5% glycerol, and 66 ng/μL poly[dI ·dC]) at 37 °C for 30 min. The binding complex was then irradiated with 15 mJ in XL-1500 UV crosslinker (Spectronic Corporation, NY, USA) for 5 min. Two microliters of 80% glycerol was added to 8 μL of the reaction mixture and electrophoresed in 1.5 % agarose gel or 6% polyacrylamide gel in 0.5× TBE buffer (89 mM Tris–borate and 2 mM EDTA) at 4 °C, 80 V for 4–5 h.

To determine the effect of antisense oligonucleotide on complex formation, a 100-fold molar excess of the appropriate unlabeled oligonucleotide was added to the reaction mixture, prior to adding labeled probe and then incubated at 37 °C for 15 min. The primer sequences used for antisense analysis were as follows: 5′-tcagttagggtagac-3′ for the template domain, 5′-gtttgctctagaat-3′ for the pseudoknot domain, and 5′-gctgacagagcccaact-3′ for the CR4–5 domain. Antisense oligonucleotide was designed to target the unpaired region in the functional domain of hTR based on its proposed secondary structure by Chen et al. [1] (Fig. 1).

2.5. Immunoprecipitation

Fifty microliters of assemble reaction mixtures was precipitated by adding anti-HA mouse monoclonal antibody (Santa Cruz Biotechnology, Inc) and 70 μl of protein G agarose (Santa Cruz Biotechnology, Inc.) in IP buffer (50 mM HEPES, pH 7.6, 100 mM NaCl, 0.1% NP-40, 0.2 mM EGTA, 1 mM MgCl$_2$, and 10% glycerol). The agarose pellet obtained was washed in 20 mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 0.2 mM EGTA, 1 mM MgCl$_2$, 0.1% NP-40, and 0.1% BSA. 2, 10, and 30 μl of beads were analyzed by TRAP, and by Western blot and Northern blot analyses, respectively.

3. Results

3.1. Minimally required hTR for telomeric DNA binding

We generated seven different hTR truncation constructs based on the secondary structure (Fig. 1A). These truncations were designed to delete the structural domains step-wise from the 3′ or 5′ end of hTR (Fig. 1B). The size and integrity of in vitro transcribed hTR were confirmed by agarose electrophoresis using a size marker (Fig. 1C).

To identify the telomere binding region of hTR, EMSA was performed using in vitro transcribed hTR and the single strand telomeric repeat sequence (TTAGGG)$_n$. At first, various lengths of (TTAGGG)$_n$ were added to the reaction to define the effective telomeric length on hTR–telomeric DNA complex formation (Fig. 2A). A strong complex was observed only with 18mer (TTAGGG)$_3$ (lane 2). Shorter (lane1) or longer (lanes 3–5) telomeric sequences than 18mer did not form any detectable complexes. This result suggests that the length of the telo-
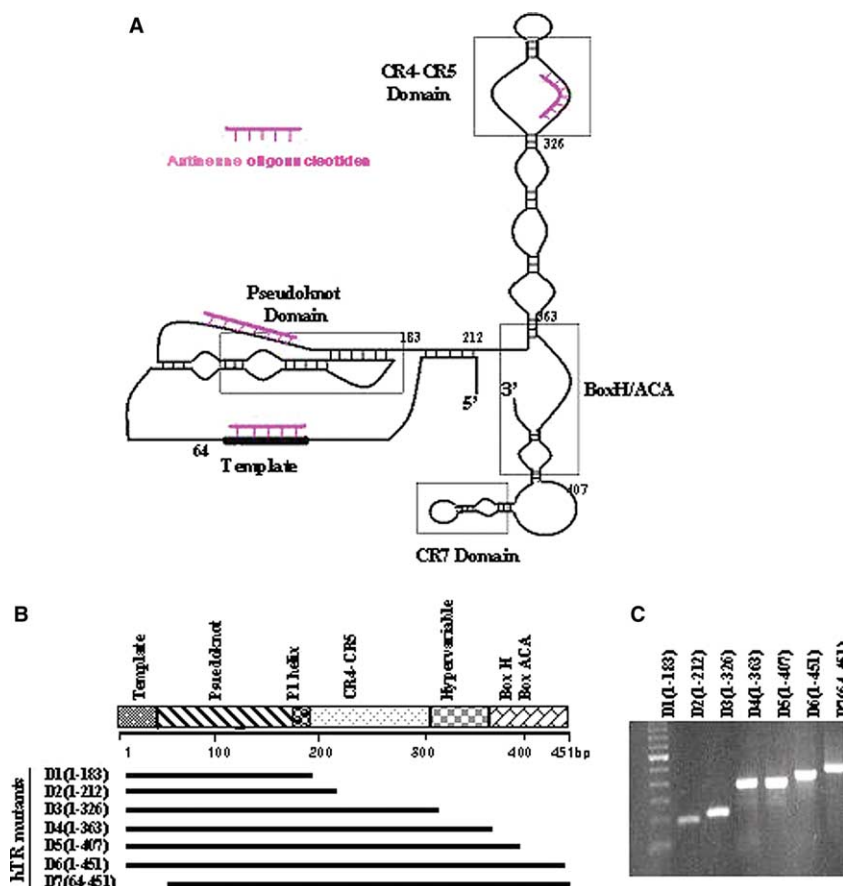

Fig. 1. Secondary structure of hTR and schematic representation of hTR mutants. (A) The secondary structure of hTR is composed of a template sequence and several conserved domains including pseudoknot, CR4–5, Box H/ACA, and the CR7 domains (in box). Red regions showed the location of antisense oligonucleotide against template, pseudoknot, and CR4–5 domains, respectively. (B) hTR mutant constructs are represented with the conserved structural domains. (C) hTR mutants were in vitro transcribed and electrophoresed to verify the accurate molecular size and amount.
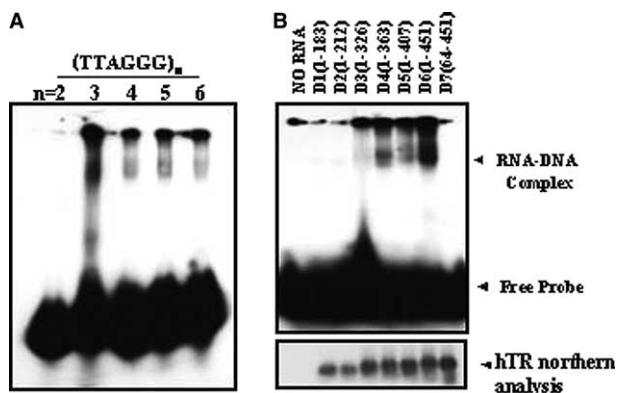
Fig. 2. Complex formation between in vitro transcribed hTR and telomeric DNA. (A) The effect of telomeric DNA length on DNA–RNA complex formation was evaluated by different lengths of ^{32}P-labeled (TTAGGG)$_n$ ($n = 2$, lane 1; $n = 3$, lane 2; $n = 4$, lane 3; $n = 5$, lane 4; $n = 6$, lane 5). (B) EMSA was carried out using ^{32}P-labeled telomeric DNA, (TTAGGG)$_3$ and various truncated hTR mutants transcribed in vitro. One picomole of ^{32}P-labeled probe was incubated without (lane 1) or with (lanes 2–8) the truncated hTR mutants. Reactions were visualized by autoradiography (top). Isolated hTR mutants were hybridized with ^{32}P-labeled oligonucleotides complementary to hTR nt+164 to nt+183 (bottom).

meric sequences is critical for binding between hTR and telomeric DNA.

We then identified the minimally required structural domains of hTR for telomere binding using hTR mutants (Fig. 2B, top). A weak binding complex was observed with D1 (1–

183) and D2 (1–212) mutants. The RNA–DNA complex formation increased gradually from D3 (1–326) to D6 (1–451). Mutants D3 (1–326), D4 (1–363) and D5 (1–407) showed 13.2%, 27.4% and 14.6% of the telomeric DNA binding activity of the wild-type D6, respectively. In contrast, D7 (64–451) did not form a complex with telomeric DNA. These EMSA results suggest that the template sequences of hTR play an essential role in binding with telomeric DNA and that the minimally required hTR for this binding is a 326 nt fragment containing the template, pseudoknot, and CR4–5 domains. To monitor the amount and integrity of the input hTR mutants in the binding assay, we isolated RNA from mixtures after the binding reaction and performed Northern blot hybridization with a hTR-specific probe (Fig. 2B, bottom). The levels and integrities of hTR mutants in the binding assay were not different from each other.

3.2. Minimally required hTR for telomerase reconstitution

To analyze the ability of hTR mutants in reconstituting active telomerase, we performed in vitro reconstitution assay with RRL system expressing hTERT. Telomerase activity increased gradually on adding two essential components of telomerase, hTR and hTERT, in a dose-dependent manner (Fig. 3A). We optimized in vitro telomerase reconstitution by adding 150 ng of each hTR transcript, 2 μL of RRL expressing hTERT, and 2 μL of fresh RRL. D1, D2 and D7 showed no telomerase activity, whereas D3, D4 and D5 showed 46%, 58.0%, and 32.0% of the telomerase activity of the wild-type D6, respectively (Fig. 3B). The results showed that 326 nt of
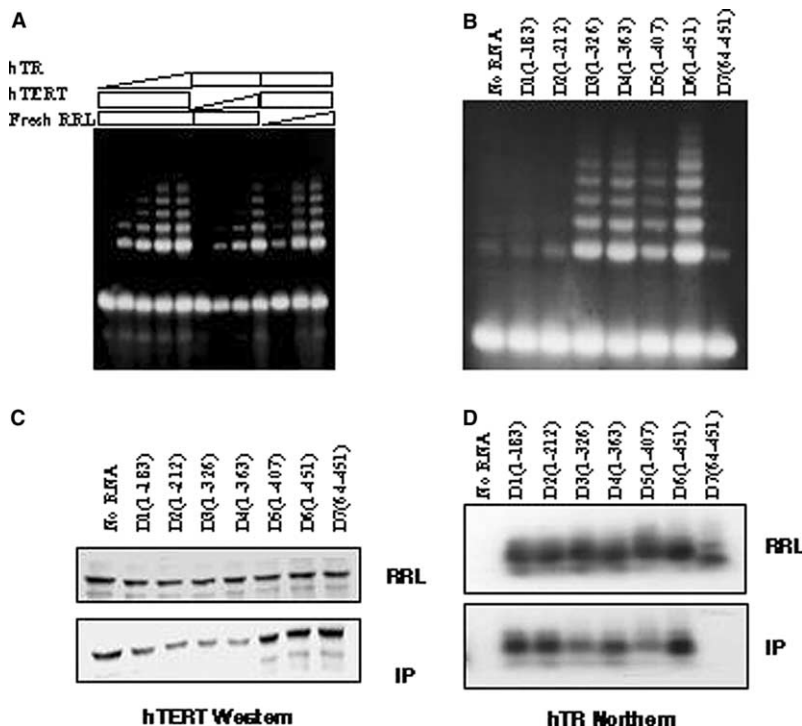

Fig. 3. In vitro reconstitution of telomerase activity. (A) The in vitro reconstitution of telomerase was evaluated with various amounts of hTR, hTERT, and fresh RRL. Lanes 1–5: 0, 10, 50, 150, 300 ng of hTR; lanes 6–9: 0, 0.5, 1, 2 μL of hTERT synthesized in vitro; lanes 10–12: 0, 1, 2 μL of fresh RRL. (B) Different mutants were reconstituted with hTERT containing HA tag and were subjected to anti-HA immunoprecipitation. The telomerase activity of the immunoprecipitate was determined by TRAP assay. (C) Before (top) or after (bottom) immunoprecipitation, Western blot analysis was performed with anti-HA monoclonal antibody. (D) Before (top) or after (bottom) immunoprecipitation, isolated RNA was subjected to Northern blot hybridization analysis with an hTR-specific probe.

hTR is essential for the telomerase activity in the RRL reconstitution system. This minimal region for telomerase activity was concordant with minimal region required for telomere binding.

3.3. Minimally required hTR for hTERT binding

To evaluate the ability of hTR mutants to associate with hTERT, each assemble reaction mixture was immunoprecipitated with HA-antibody to HA tag located at the carboxy terminus of hTERT. And the immunoprecipitate was subjected to Western blot (Fig. 3C) and Northern blot analyses (Fig. 3D). The amounts of total and precipitated hTERT were similar for all fractions (Fig. 3C). Despite the same amount of input hTR mutants in all assemble reaction fractions, mutant D7 spanning nucleotides 64–451 did not co-immunoprecipitate with hTERT. These results suggest that 1–64 nt structural region of hTR plays a critical role in the binding with the catalytic subunit of telomerase. Therefore, the mutant D1 spanning 1–183 was sufficient for the interaction with hTERT. The relative ratios of the enzymatic activities, telomeric DNA and hTERT binding capacities of each hTR mutant are summarized in Table 1. These results show that different structural regions of hTR are required for DNA (telomere) binding and for protein (hTERT) binding.

3.4. Comparison of structural domains of hTR for telomere binding and telomerase activity by antisense oligonucleotide

As shown in Figs. 2 and 3, minimal sequence of hTR required for telomere binding and telomerase activity contains three functional domains, i.e., the template, pseudoknot, and CR4–5 domains. To evaluate the role of each domain, antisense oligonucleotides were designed to target the unpaired region of the template, pseudoknot, or CR4–5 domains. Fig. 4A 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 competed by adding a non-labeled homologous competitor (lane 2), but was not inhibited by any of the non-specific primers of equal molecular weight $[(TGTGAG)_3$, lane 3 and $(TTGGAG)_3$, lane 4]. These findings suggest that the formation of the hTR–telomere complex is mediated by sequence-specific recognition. Anti-template oligonucleotides completely abolished the RNA–DNA binding (lane 5), whereas anti-CR4–5 oligonucleotides reduced RNA–DNA binding by only 4% (lane 7). On the other hand,
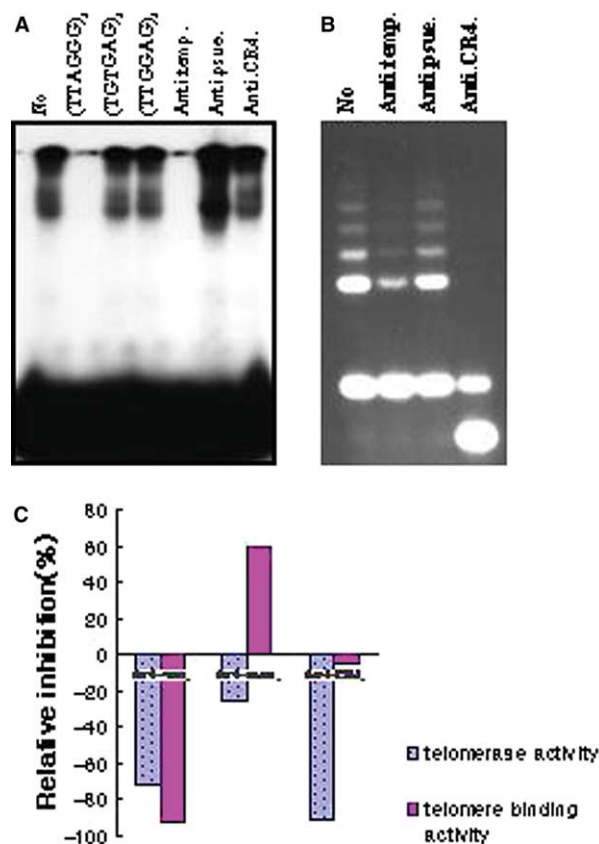

Fig. 4. Effect of antisense oligonucleotides on the formation of the RNA–DNA complex and telomerase activity. (A) EMSA was performed in the absence (lane 1) or in the presence (lanes 2–7) of competitive primers or antisense oligonucleotides. hTR was incubated with a non-labeled homologous competitor (lane 2), non-specific primer $[(TGTGAG)_3$, lane 3; $(TTGGAG)_3$, lane 4], anti-template oligonucleotides (lane 5), anti-pseudoknot oligonucleotides (lane 6), or anti-CR4–5 oligonucleotides (lane 7) prior to adding the ^{32}P-labeled probe. (B) After incubating hTR with antisense oligonucleotides, hTERT was added to the reaction and a TRAP assay was performed. (C) Relative quantitation of the degree of inhibition by antisense oligonucleotides.

anti-pseudoknot oligonucleotide enhanced the complex formation (lane 6). We also analyzed the antisense oligonucleotides effect on telomerase activity (Fig. 4B). Anti-CR4–5 and anti-template oligonucleotides effectively inhibited telomerase

Table 1
Summary of hTERT biding activity, telomeric DNA binding ability, and telomerase activity of hTR truncations

Telomerase RNA	Characterization of structure	hTERT binding	Telomeric DNA binding	Telomerase activity
hTR D1 (1–183)	Truncated 3′ terminal 268 nt; partly disrupted P1 helix, deleted CR4–5 domain, BoxH/ACA and CR7 domain	+++++	−	−
hTR D2 (1–212)	Truncated 3′ terminal 239 nt; deleted BoxH/ACA and CR7domain	+++++	−	−
hTR D3 (1–326)	Truncated 3′ terminal 125 nt; partly disrupted hypervariable region, deleted BoxH/ACA and CR7 domain	++++	+	+++
hTR D4 (l–363)	Truncated 3′ terminal 88 nt; deleted BoxH/ACA and CR7domain	+++++	++	+++
hTR D5 (1–407)	Truncated 3′ terminal 44 nt; partial disrupted BoxH/ACA, deleted CR7 domain	++++	+	++
hTR D6 (1–451)	Full length, wild type	+++++	+++++	+++++
hTR D7 (64–451)	Truncated 5′ terminal 64 nt; deleted template region	−	−	−

Relative activity of each mutant was determined by defining the relative level of activity against wild-type hTR taken as 100%. The activity of all mutants is represented symbolically as a relative range: +++++, greater than 80%; ++++, between 80% and 61%; +++, between 60% and 41%; ++, between 40+% and 21%; +, between 20% and 1%; −, less than 1%.

activity by 90.6% and 70.4%, respectively. Anti-pseudoknot oligonucleotide also reduced telomerase activity by 25.9%.

4. Discussion

The length of the telomeric sequences was found to be critical for in vitro binding between hTR and telomeric DNA. Only (TTAGGG)₃, 18mer formed a stable complex with hTR. Telomeric DNA shorter or longer than 18mer did not form a strong complex, which suggests that telomeric sequences shorter than 18mer are too short to form a stable base pair with hTR. In contrast, longer sequences than 18mer had been suggested to form secondary structures by themselves, e.g., the G-quartet [10,11].

It was suggested that nucleotides +33 and +325 of hTR containing the template, pseudoknot, and CR4–5 domains represent the minimal requirement for telomerase activity in RRL system. In particular, the integrities of CR4–5 and of the pseudoknot structure were found to be critical for the reconstitution of telomerase activity in studies using truncated or sequence-substituted hTR mutants [3,4]. Direct interaction of hTR–hTERT was also investigated by using an in vitro telomerase reconstitution assay. Autexier et al. [3] reported that 159 nt of hTR is sufficient for a stable interaction with hTERT and found that two distinct hTERT-binding sites existed at nucleotides +33 and +147 and +164 and +325. However, no investigation has been conducted on the role of the hTR structural domains in terms of its binding with telomeric DNA. Our results showed that the minimal region of hTR required for telomere binding is a 326 nt fragment of hTR, which contains the template, pseudoknot and CR4–5 domains. We also confirmed that this 326 nt fragment of hTR is critical for the expression of telomerase activity. On the contrary, a small fragment (183 nt) of hTR was sufficient for the interaction with hTERT. Hence, hTR–DNA (telomere) binding needs more stable structure of hTR than hTR–protein (hTERT) binding in terms of telomerase activity.

Template domain has known to fold into a long hairpin structure, in which the template sequence occupies a readily accessible position [10,12]. Neither in vivo nor in vitro hTR mapping has confirmed formation of a stable psuedoknot helix. Rather, it has suggested that the pseudoknot domain exists in two alternative states of nearly equal stability in solution. One is the previously proposed pseudoknot formed by pairing with P3 with template region and the other is a structural P2b loop alone [13]. Disruption of the pseudoknot domain by antisense oligonucleotide reduced telomerase activity, but enhanced telomere binding in our study. This finding can be partly explained by equilibrium shift phenomenon. Theimer et al. [14] showed that mutation in the pseudoknot domain results in a shift in the equilibrium toward the hairpin form, primarily due to stabilization of the pseudoknot. With this hairpin form excessiveness, the binding capacity with telomere may increase even with loss of telomerase activity. In contrast to pseudoknot domain, disruption of template region by antisense oligoneucleotide completely blocked telomere binding and thus inhibited telomerase activity reconstruction as we assumed based on previous data.

The role of the CR4–5 domain has not been elucidated, but a truncated mutant without the CR4–5 domain did not have tel-

omerase activity [3]. Stable base pairing of the P6.1 stem was required for the RNA–protein interaction between the CR4–5 domain and hTERT [15]. Moreover, mutation of unpaired nucleotides stem–loop of activation domain abolished enzymatic activity [16]. We found that antisense oligonucleotide targeting the CR4–5 domain only partially inhibited telomeric DNA–hTR binding, but completely inhibited its enzymatic activity, suggesting that a minor structural change of its region can be a good strategy for telomerase inhibition. In previous study, two hTR mutations were found which allowed RNA–protein binding but significantly impaired telomerase activity [15]. Similar to this, our results showed that antisense oligonucleotide targeting the pseudoknot and the CR4–5 domain completely inhibited telomerase activity, but did not inhibit telomere binding. This finding suggests that the three structural domains (template, pseudoknot, and CR4–5 domains) play distinct functional roles in terms of the activation of telomerase and telomere binding. We found that hammerhead ribozyme targeting the template region of hTR efficiently cleaved hTR in vitro, and stable transfectants of this ribozyme induced the degradation of target hTR RNA and attenuated telomerase activity in MCF-7 cells. Moreover, the ribozyme R1 transfectant displayed a significant telomere shortening and a lower proliferation rate than parental cells. Clones with reduced proliferation capacity showed enlarged senescence-like shapes or highly differentiated dendritic morphologies of apoptosis. Ribozyme targeting CR4–5 domain showed weak cleavage activity of the target site [17].

It is suggested that each functional domain of hTR may perform a different regulatory function in terms of telomere binding and telomerase activity.

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