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Structure, Interactions and Effects on Activity of the 5′-terminal Region of Human telomerase RNA

Xianglan Li, Hidetoshi Nishizuka, Kota Tsutsumi, Yuka Imai, Yasuyuki Kurihara and Seiichi Uesugi*

Department of Environment and Natural Sciences, Graduate School of Environment and Information Sciences, Yokohama National University, 79-7 Tokiwadai, Hodogaya-ku, Yokohama 240-8501, Japan

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Telomerase is an enzyme that catalyzes addition of telomeric repeat sequences to the 3′-termini of eukaryotic chromosome DNA. The catalytic core of telomerase consists of a protein component, telomerase reverse transcriptase (TERT), for the catalysis and an RNA component, telomerase RNA (TR), containing the template for the sequence. Human telomerase RNA (hTR) consists of 451 nucleotides (nt) and contains consecutive G-stretches in the 5′-terminal region. We examined the effects of the 5′-terminal sequence (nt 1–17) in hTR, which is assumed to be a single-stranded region (region 1), on interaction and telomerase activity *in vitro*. Mutation and binding experiments for hTR and its variants suggest that region 1 has repressive effects on telomerase activity by interaction with the region(s) in the 3′-half part. We prepared various hTR variants with mutations in region 1 and two possible target regions (region 2: nt 229–244; region 3: nt 284–297). Studies on these variants showed that region 1 can interact with regions 2 and 3 and the interactions between regions 1 and 3 may contribute to the repressive effects of region 1. We found that a mutation in region 2 markedly enhances telomerase activity. We also found that some deletion and sequence mutations in region 1 enhance the activity.

Key words: gel mobility shift assay, mutation, RNA–RNA interaction, RNA structure, telomerase RNA.

Abbreviations: aa, amino acid; nt, nucleotide; NTP, nucleoside triphosphate; PCR, Polymerase chain reaction.

INTRODUCTION

Telomeres are the protective termini of eukaryotic chromosomes comprised of repeated DNA sequences and their associated proteins. The telomere DNA is shortened during replication with each cell division. Telomerase is an enzyme that maintains the telomere DNA length by adding nucleotide residues to the 3′-ends of the DNA strands (*1*, *2*). Telomerase activity is usually not detected in normal cells but is detected in highly proliferating cells such as cancer cells (*3*).

The catalytic core of telomerase consists of an RNA component (telomerase RNA: TR), which contains the template sequence for telomeric repeat sequences (GGTTAG in vertebrates), and a protein component (telomerase reverse transcriptase: TERT), which acts as reverse transcriptase (RNA-dependent DNA polymerase) (*4–6*). Although many other proteins are associated with telomerase activity *in vivo*, it is known that the reconstituted complex of TR and TERT, which are prepared *in vitro*, shows telomerase activity (*7–9*).

TERT is a large protein of 100–130 kDa [human TERT (hTERT): 1,132 aa] and contains highly conserved amino acid sequence motifs specific for reverse transcriptase (multiple RT-motifs) and for telomerase (T-motif) (*10*). TRs of vertebrates consist of 400–600 ribonucleotide residues [human TR (hTR): 451 nt] and their sequences are considerably different among species. Using phylogenetic comparative analysis, Chen *et al*. showed that the secondary structure of vertebrate TR contains four conserved structural domains: core (or pseudoknot), CR4/5, box H/ACA and CR7 domains (Fig. 1) (*11*). The template sequence is included in the core domain (*12*). It was experimentally shown that all four of these domains contribute to telomerase function *in vivo* (*13*). However, human telomerase catalytic activity requires only the core and CR4/CR5 domains, each of which can bind independently to the TERT protein, *in vitro* (*9*, *14*, *15*). The box H/ACA and CR7 domains are necessary for TR stability and localization in the nucleus (*16*, *17*).

The CR4/CR5 domain of hTR is necessary for assembly and activity of telomerase *in vivo* and *in vitro* (*18*). A stem (P6.1)-loop (L6.1) structure has been found in the J6/5 region (*11*) of this domain (*15*). The sequence of the P6.1 stem-loop and the stem structure are important for binding to TERT and telomerase activity (*15*, *19*, *20*). It has been shown that in the P6 and L6 regions (*11*) of the CR4/CR5 domain, which is just above the P6.1 stem-loop, the sequence and structure of the J6 internal loop (*21*) are also important for interaction with TERT (*9*, *21*, *22*).

The sequence and length of the 5′-terminal region in vertebrates, which is upstream of the template region, are different among species. In the case of hTR, there are 45 nt upstream of the template segment

*To whom correspondence should be addressed.
Tel/Fax: 045-339-4265, E-mail: siuesugi@ynu.ac.jp

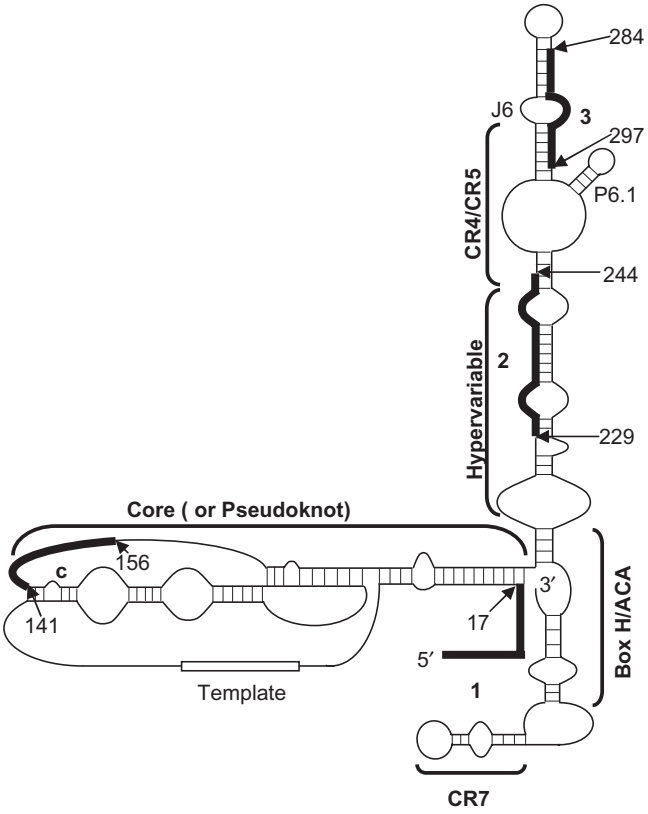

Fig. 1. **Schematic model of the secondary structure of human telomerase RNA originally proposed by Chen *et al.* (*11*).** The structure for the CR4/CR5 domain is modified according to Theimer & Feigon (*42*). The thick lines show regions 1–3 and c focused in this paper.

(5′-CUAACCCUAAC-3′) (*23*). According to the secondary structure model proposed by Chen *et al.* (*11*), the upstream region consists of a single-stranded region (nt 1–17) at the 5′-end, a region forming the P1 helix (nt 18–37), and a linker region (nt 38–45) joining the P1 helix and the template region. The stem structure of the P1 helix is important for defining the template boundary and for telomerase activity (*12*, *14*, *24*, *25*). The length but not the sequence of the linker segment determines the template boundary (*24*). The 5′-terminal region contains several stretches of consecutive G residues. Some variants of hTR, which have deletion in the 5′-terminal region, show higher telomerase activity than those without deletion in that region (*14*, *26*). In the case of mouse TR (mTR), there are only 2 nt upstream of the template segment (*27*). A minor transcript of mTR gene detected in mouse cells contains 36 nt upstream of the template region and shows very low telomerase activity (*28*). These data suggest that the 5′-terminal region of TR may affect the level of telomerase activity by intramolecular RNA-RNA and/or RNA-protein interactions.

It has been shown by *in vitro* studies that combined fragments of the CR4/CR5 domain and the core domain, which includes the template and the pseudoknot regions, can reconstitute telomerase activity in the presence of TERT (*9*). Recently, intermolecular interactions between a fragment containing the template region in the core domain and a fragment containing the P6.1 region in the CR4/CR5 domain have also been characterized through mobility shift assays, mutational analysis and UV cross-linking analysis (*29*). However intramolecular tertiary interactions within an hTR molecule and their effects on telomerase activity are not well understood. In this paper, we examined intramolecular interactions and their effects on telomerase activity of the 5′-terminal single-stranded region (region 1) in hTR. The results suggest that the presence of region 1 reduces telomerase activity and region 1 interacts with specific segments in the hypervariable region (region 2) and CR4/CR5 domain (region 3). Mutations of hTR to enable region 1 to stably interact with region 2 and mutations to remove possible interaction of region 1 with other regions enhanced telomerase activity. These results suggest that intramolecular tertiary interactions in hTR can regulate the level of telomerase activity.

MATERIALS AND METHODS

Preparation of DNA Templates for T7 RNA Polymerase—Preparation of a template DNA for **R1M** fragment

The template for **R1M** fragment was designed so that the initial transcript contains a sequence of a hammerhead ribozyme and is cleaved to give a **R1M** fragment (*30*, *31*). The template DNA was prepared by the PCR (Polymerase Chain Reaction) method using *Taq* DNA polymerase and primers for a sense strand (5′GTAA TACGACTCACTATAGGAAAGTATAGAGTGTCTAGCGT CTAGTCGC3′) and an antisense strand (5′AGAGTGT TTCGGCCTTTCGGCCTCATCAGTAGCGACTAGACGCT AGACA3′).

Preparation of template DNAs for variants of hTR

The template DNAs for deletion variants of hTR were prepared by the PCR method using a plasmid pUC19-hTR, which contains cDNA of hTR at *Eco*RI-*Pst*I sites, and appropriate primers shown in Table 1.

For preparation of template DNAs for other regional variants, we adopted the overlapping PCR technique with pUC19-hTR and SP *Taq* DNA polymerase (*32*, *33*). For example, the template DNA for hTR2M, which contains complementary mutations in the segments 229–242 and 331–343, was prepared by two steps of the overlapping PCR. The cDNA for an hTR variant, which contains a mutation in the segment (nt 229–242), was prepared at first using combinations of forward and reverse primers (Eco-T7 and HTR2M251R; HTR2M222F and Pst-HTR451R). Then a second mutation in the segment (nt 331–343) was introduced to the DNA obtained above using combinations of forward and reverse primers (Eco-T7 and HTR2M351R; HTR2M321F and Pst-HTR451R). After appropriate selection, the plasmid pUC19-hTR2M was isolated and the sequence was confirmed. The plasmids for other variants, hTR3M and hTR2M3M, were prepared in a similar manner. These plasmids were cut with *Pst*I to give the proper templates for transcription by T7 RNA polymerase

Table 1. **Primers for amplifying hTR sequences.**

Primer	Sequence
Eco-T7[a]	5′GGCGAATTCTAATACGACTCACTATA3′
HTRW△20F	5′CCGGAATTCTAATACGACTCACTATAGGGAGGGGTGGTGGCCATTT3′
HTRW△7F	5′GGCGAATTCTAATACGACTCACTATAGGAGGGTGGGCCTGGGAG3′
HTRW△14F	5′GGCGAATTCTAATACGACTCACTATAGGGCCTGGGAGGGGTGGTG3′
HTR17AF	5′ATGCGAATTCTAATACGACTCACTATAGAGTTGCAGAGAGTGAGCCTGGG3′
HTR1M'F	5′GGCGAATTCTAATACGACTCACTATAGGGTTGCTCACCCTGGGCCTGGGA3′
HTR1MF	5′GGCGAATTCTAATACGACTCACTATAGGAAAGTATAGAGTGTCCCTGGGA3′
HTR2M222F	5′TCGCCTGGACACTCTATATTTCCCGCCTGGAGGCCGCGG3′
HTR2M321F	5′GCGGGTCTCATATAGAGCAGTCCGAGGTTCAGGCCTTTCAG3′
HTR3M246F	5′GCCTGGAGGCCGCCGGAAACCCGGGGCTTCTCCGA3′
HTR3M275F	5′CTCCGAGCACTCTATAGTTTCCGGCGAAGAGTTGGGCTCTG3′
HTR207F	5′GGCGAATTCTAATACGACTCACTATAGGGACCTGCGGCGGGTCG3′
HTR451R	5′GCATGTGTGAGCCGAGTCCTG3′
Pst-HTR451R[b]	5′GGCCTGCAGCATGTGTGAGCCG3′
HTR2M251R	5′CCAGGCGGGAAATATAGAGTGTCCAGGCGACCCGCCGCAG3′
HTR2M351R	5′GAACCTCGGACTGCTCTATATGAGACCCGCGGCTGACAGAGCC3′
HTR3M275R	5′GAAGCCCCGGGTTTCCGGCGGCCTCCAGGCGGG3′
HTR3M304R	5′TCTTCGCCGGAAACTATAGAGTGCTCGGAGAAGCCCCGGGTT3′
HTR209R	5′CCCCGGGAGGGGCGAAC3′

[a]This primer contains sequences for *Eco*RI recognition and T7 promoter. [b]This primer contains sequence for *Pst*I reconition. The mutated bases or sequences are underlined.

and isolated by ethanol precipitation after phenol-chloroform extraction.

Preparation of RNA—Some short RNA oligomers including **R17** fragment were chemically synthesized by a DNA/RNA synthesizer and purified by 7 M urea-16% polyacrylamide gel electrophoresis after deprotection as described previously (*34*). Other longer RNAs were prepared by transcription with T7 RNA polymerase using appropriate DNA templates under the conditions as previously described with some modification (*35*). A typical reaction mixture (100 μl) contained T7 RNA polymerase, template DNA (5 μg), 7.5 mM NTPs, 35 mM $MgCl_2$, 40 mM Tris-HCl (pH 8.0), 10 mM DTT, 2 mM spermidine, 20 mM GMP and RNase inhibitor (20 U) and was incubated at 37°C for 4 h. The mixture was added with 7M urea (70 μl), heated at 90°C for 1.5 min, and placed on ice for 5 min. The product RNA was separated by 7 M urea-20% or –6% polyacrylamide gel electrophoresis, extracted from the gel with 0.3 M sodium acetate, 0.01% SDS, 25 mM Tris-HCl (pH 8.0), 1 mM EDTA for 4 h ∼ overnight (*36*) and isolated by ethanol precipitation. The precipitates were dissolved in RNase-free water.

Measurement of CD Spectra—**R17** (40 μM) in 10 mM sodium phosphate buffer (pH 6.5) (300 μl) was annealed by heating at 90°C for 3 min and gradual cooling to room temperature. CD spectra were recorded with a JASCO J-720 spectrometer equipped with a temperature-control unit and scanning program. The spectra were measured at 5°C and at various K^+ ion concentrations using a 1-mm cell. The CD intensities were expressed in [θ] per residue. The melting temperature (T_m) was measured by monitoring [θ] at 265 nm with increasing temperature at a rate of 50°C/h.

Assay of Telomerase Activity—Human telomerase reverse transcriptase (hTERT) was expressed in the rabbit reticulocyte lysate (RRL) (Promega) system by incubation of a mixture (2.5 μl) of the lysate (60–70%, v/v) and a plasmid pcDNA-hTERTn2 (60–70 ng), which was cut with *Not*I, at 30°C for 90 min. hTR or its variant (200 ng), which was transcribed *in vitro* by T7 RNA polymerase, and an additional volume of fresh RRL (1.5 μl) were added to the mixture (*37*). The reaction mixture was incubated at 30°C for 90 min. A portion of the solution (1 μl) was removed and subjected to telomerase assay by using TeloChaser kit (TOYOBO). The primer extension reaction was performed by incubation at 30°C for 60 min under the conditions with or without added K^+ or Na^+ ions (150 mM). The products were amplified by PCR with hot-start at 95°C (95°C for 30 s, 68°C for 30 s, 72°C for 45 s; 26 cycles) and analysed by native 10% polyacrylamide gel electrophoresis using 0.7 × TBE buffer at room temperature (100 V for 10 min then 200 V for 65 min). The product bands were stained with SYBR Green for 30 min and analysed by FLA-2000 (Fuji Film).

Preparation of 5′-^{32}P-labelled RNA—RNA transcript (300 pmol) was treated with *Escherichia coli* alkaline phosphatase (8 U) at 37°C for 1 h, then with phenol-chloroform and isolated by ethanol precipitation. The dephosphorylated RNA (20 pmol) was treated with [γ-^{32}P]ATP (2 μl) (ICN) and T4 polynucleotide kinase (9 U) (TOYOBO) at 37°C for 1 h. After addition of 9 M urea (10 μl), the reaction mixture was subjected to 7 M urea-20% polyacrylamide gel electrophoresis at room temperature and 45 mA for 30 min. The product band was detected with an X-ray film. The product was extracted from gel and isolated by ethanol precipitation.

Gel Mobility Shift Analysis—5′-^{32}P-labelled RNA fragment (2 nM, 2 μl) and cold RNA (40–4000 nM, 2 μl) were separately annealed by heating at 90°C for 1.5 min, placed on ice for 5 min and mixed. Binding buffer (4 μl) was added to the mixture to give a solution in 50 mM sodium phosphate buffer (pH 7.5), 40 mM KCl, 0.1 mM $MgCl_2$. The solution was kept at 37°C for 30 min and

loading buffer was added to make a loading solution (10 μl) in 50 mM sodium phosphate buffer (pH 7.5), 40 mM KCl, 0.1 mM $MgCl_2$, 8% glycerol. The solution was applied onto native 6% polyacrylamide gel (acrylamide/bisacrylamide ratio of 29:1) containing 5 mM $MgCl_2$ and subjected to electrophoresis in 89 mM Tris-borate, 5 mM $MgCl_2$ at room temperature and 200 V for 1.5 h. The gel was treated with an imaging plate for 4 h overnight and analysed by FLA2000. In the case of competition experiments, DNA oligomer was added to the solution of **R17** and hTR pre-incubated at 37°C for 30 min and the mixture was further incubated at 37°C for 30 min prior to electrophoresis.

RESULTS AND DISCUSSION

G-rich RNA Fragment of 5′-terminal Sequence of hTR (nt 1–17) Forms G-quadruplex Structure—The base sequence of region 1 in hTR (nt 1–17: 5′GGGUUGCGGAGGGUGGG-3′) contains several stretches of consecutive G. It is well known that this kind of G-rich sequence can easily form a G-quadruplex structure. To confirm this possibility, we prepared a 17-mer RNA fragment (**R17**) containing the sequence of region 1 and measured its CD spectra (Fig. 2). **R17** showed a large positive band around 265 nm and a moderate negative band around 245 nm even in the absence of K^+ ions. This CD pattern is characteristic of a parallel G-quadruplex structure (*38*, *39*). Moreover, the positive band around 265 nm increased with increasing concentration of K^+ ion, known to stabilize G-quadruplex structure. R17 showed sharp melting curves with T_ms at 44°C, 57°C and 81°C in the absence of added metal ions, at 150 mM NaCl and 150 mM KCl, respectively. A 17-mer RNA fragment (**R17A**: 5′GAGUUGCAGAGAGUGAG3′) containing G-to-A mutations to interrupt the consecutive G stretches of **R17** was also prepared and its CD spectra were measured. **R17A** showed only a small and broad positive band ($[\theta] < 10^4$) and almost no change upon K^+ ion addition (up to 150 mM) suggesting lack of quadruplex formation (data not shown).

Repressive Effect of Region 1 on Telomerase Activity is Not Due to Formation of G-quadruplex—The above results suggest that the sequence of region 1 in hTR has an intrinsic nature to form a G-quadruplex. Effects of region 1 on telomerase activity in this context were examined using hTR variants with modification in this region. The variants with the same sequence as that of **R17A** in region 1 (hTR17A) and with deletion of nt 1–20 (hTRΔ20) were prepared by *in vitro* transcription with T7 RNA polymerase. Telomerase was reconstituted by adding each hTR to hTERT produced by *in vitro* translation in the rabbit reticulocyte system. The primer extension reactions were carried out without or with added metal ions (Na^+ or K^+, 150 mM), which are known to stabilize G-quadruplex structures. After the primer extension reaction, the products were amplified by the stretch PCR method and analysed by native polyacrylamide gel electrophoresis (Fig. 3). A ladder of bands detected represents DNA duplexes containing telomeric products, in which the chain lengths are different by 6 bp. The chain of the primer is extended by repeated addition of GGTTAG sequences. The lowest band of the ladder is of 82 bp which means that the primer has been extended by 25 nt (four times six plus one).

Although addition of metal ions markedly reduced telomerase activity for all three hTR derivatives, the wild-type hTR (hTRW) and hTR17A showed about the same activity but hTRΔ20 showed considerably higher activity than those of hTRW and hTR17A under all three conditions examined (Fig. 3A). These results suggest that repression of telomerase activity by the presence of region 1 in hTR is not due to formation of an

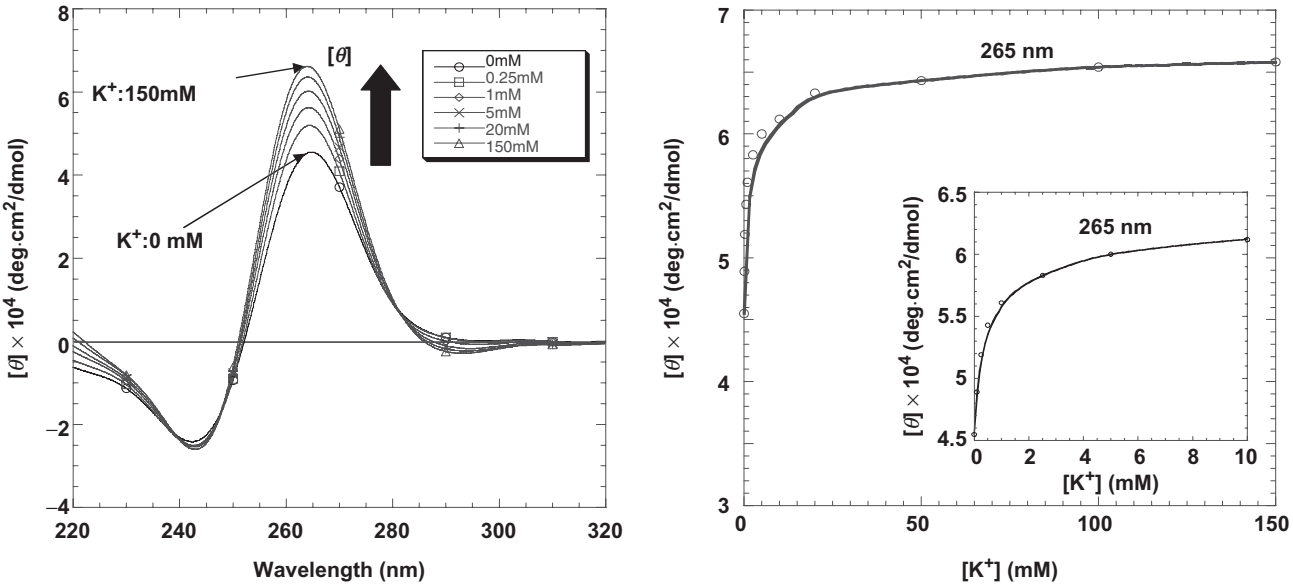

Fig. 2. **(A) CD spectra of R17 fragment (40 μM) in 10 mM sodium phosphate buffer (pH 6.5) with added K^+ ions at 5°C.** (B) Dependence of [θ] at 265 nm on K^+ concentration.

unfavourable G-quadruplex because hTR17A, in which the consecutive G stretches in hTRW are interrupted by G-to-A mutations, shows about the same activity as that of hTRW even in the presence of 150 mM K^+ ions. The reduced activity observed when the primer extension reaction is carried out in the presence of metal ions may be due to G-quadruplex formation of the primer DNA (38 nt) containing -$(TTGGGG)_3$TTG sequence at the 3′-end. Similar phenomena have been observed for human telomerase systems, in which the processivity of telomerase is impared at high KCl concentration (*40*, *41*). One plausible explanation for the reduced activity of hTRW is that region 1 interacts with other parts of the molecule, interfering with a favourable conformation or preventing necessary intramolecular interactions within hTR or intermolecular interactions with hTERT.

Addition of Antisense DNA to Region 1 Enhances Telomerase Activity—Effects of addition of an antisense DNA fragment (anti-**D17**: d-CCCACCCTCCGCAACCC), which can hybridize with region 1, on telomerase activity were also examined (Fig. 3B). Addition of anti-**D17** to the reconstitution mixture markedly enhanced telomerase activity under the conditions without added metal ions even at anti-**D17**/hTRW (1:1) (Fig. 3B, lanes 2–4), though the enhancement was somewhat reduced at anti-**D17**/hTRW (1:100). This reduction may be due to that the excess anti-**D17**, which is C-rich, binds to the primer for the extension reaction, which is G-rich, and represses telomerase activity. In the presence of 150 mM K^+ ions, similar results were observed in terms of relative activity (Fig. 3B, lanes 7–9). These results are consistent with a notion that anti-**D17** binds to region 1 breaking unfavourable interactions of the region with other elements. In contrast to the above results, addition of a sense DNA fragment (sense-**D17**: d-GGGTTGCGGAGG GTGGG, 10-fold excess) significantly reduced telomerase activity (Fig. 3B, lanes 5 and 10). Since sense-**D17** did not show binding to hTR as analysed by gel mobility shift assay (data not shown), this inhibitory effect could be due to that the G-rich sense-**D17** binds to the primer anchoring site of hTERT competing with the primer of the telomerase reaction.

R17 Fragment Binds to the 3′-half Domain of hTR—To elucidate possible interaction between region 1 and other parts of hTR, we used gel mobility shift analysis. Mixtures of ^{32}P-labelled **R17** and increasing concentrations of cold hTRW (10–1000 nM) were analysed by native polyacrylamide gel electrophoresis (Fig. 4, lanes 2–4). Sharp and thick band(s) of lower mobility were observed suggesting that **R17** actually binds to hTR. It is known that *in vitro* telomerase activity can be reconstituted by combining 5′- and 3′-halves of hTR (*9*). This fact suggests that these half molecules constitute independent structural and functional domains. Next, we prepared the 5'-half molecule (nt 1–209) and 3′-half molecule (nt 207–451) of hTR and examined interactions with **R17**. Combination of **R17**-hTR(1–209) gave smear band pattern suggesting rather non-specific binding (Fig. 4, lanes 5–7). In contrast, combination of **R17**-hTR(207–451) gave sharp shifted bands suggesting specific binding (Fig. 4, lanes 8–10). These results reveal that **R17** binds more specifically and strongly to the 3′-half molecule. The results also reveal that binding affinities of **R17** to hTR and the 3′-half molecule are about the same with K_d around 100 nM suggesting that major binding site of **R17** is in the 3′-half domain of hTR. The results are also consistent with the tertiary folding model of hTR structure proposed by Ueda and Roberts,

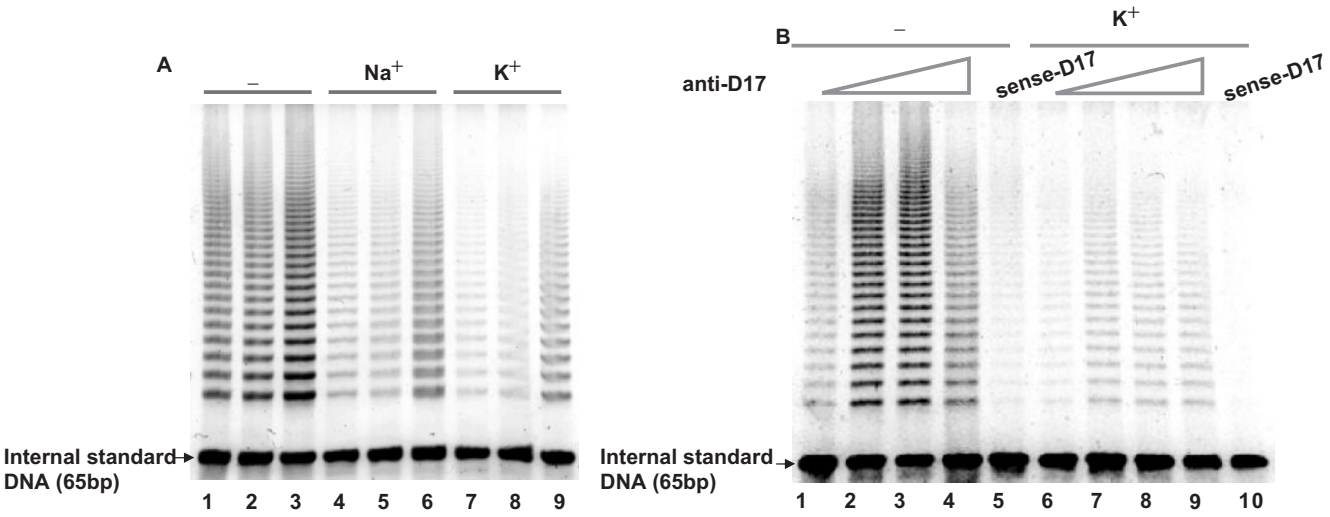

Fig. 3. **(A) Effects of the 5′-terminal sequence and deletion variants of hTR on telomerase activity assayed by the stretch PCR method.** The PCR products were analyzed by polyacrylamide gel electrophoresis. The primer extension reactions catalyzed by reconstituted telomerase for hTRW (lanes 1, 4 and 7), hTR17A (lanes 2, 5 and 8/) and hTRΔ20 (lanes 3, 6 and 9) were carried out without added metal ions (lanes 1–3), with added Na^+ ions (150 mM, lanes 4–6) or K^+ ions (150 mM, lanes 7–9). (B) Effects of addition of antisense DNA fragment (anti-**D17**) or sense DNA fragment (sense-**D17**) on telomerase activity of hTRW. The primer extension reactions were carried out without added ions (lanes 1–5) or with added K^+ ions (150 mM, lanes 6–10). Anti-**D17** was added at DNA/hTRW ratios: 0 (lanes 1 and 6); 1 (lanes 2 and 7); 10 (lanes 3 and 8); 100 (lanes 4 and 9). Sense-**D17** was added at a DNA/hTRW ratio of 10.

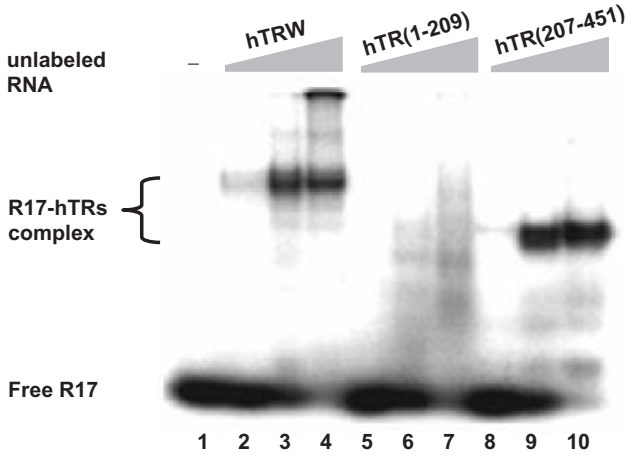

Fig. 4. **Binding experiment of R17 to hTRW and its 5′- and 3′-half molecules, hTR(1–209) and hTR(207–451), as assayed by the gel mobility shift analysis.** Increasing amounts (10–1000 nM) of unlabelled hTRW (lanes 2–4), hTR(1–209) (lanes 5–7) or hTR(207–451) (lanes 8–10) were added to ^{32}P-labelled **R17** and the mixture was analysed by polyacrylamide gel electrophoresis at room temperature. Lane 1: no added unlabelled RNA; lanes 2, 5 and 8: 10 nM RNA; lanes 3, 6 and 9: 100 nM RNA; lanes 4, 7 and 10: 1000 nM RNA.

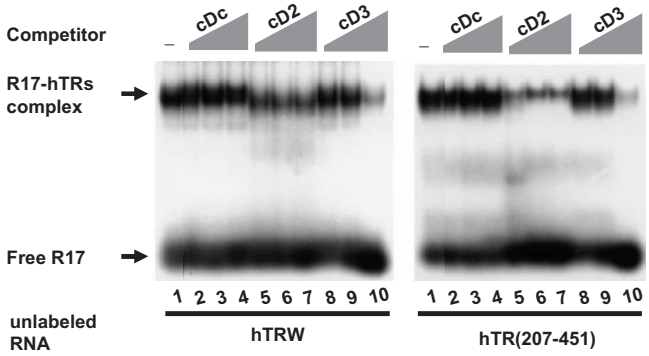

Fig. 5. **Competition experiments between competitor DNA fragments and R17-hTR complexes.** To the mixture of ^{32}P-labelled **R17** and hTRW (100 nM) or hTR(207–451) (100 nM), each competitor DNA fragment (**cDc**: lanes 2–4; **cD2**: lanes 5–7; **cD3**: lanes 8–10) was added and the mixture was analysed by the gel mobility shift assay. The competitor fragments were added at DNA/hTR ratios: 0 (lane 1); 1 (lanes 2, 5 and 8); 10 (lanes 3, 6 and 9); 100 (lanes 4, 7 and 10).

in which the template region and P6.1 in the CR4/CR5 domain are in close proximity (*29*). Region 1 can be expected to be located in the proximity of the CR4/CR5 domain favourable for intramolecular association.

Competitor DNA Binding to hTRW and hTR(207–451) Complexed with R17—To identify the binding site of **R17** in the 3′-half domain, we searched for possible binding sequences in the 3′-half domain with long complementary segments for **R17**, using a multiple sequence alignment program, ClustalW (http://www.ebi.ac.uk/clustalw/). The best candidate obtained was a region (region 2: nt 229–244; 5′CCCAGCCCCCGAACCC3′) in the hypervariable, paired region next to the CR4/CR5 domain (*11*) (Fig. 6B). The second candidate obtained by the alignment with nt 1–14 of **R17** was a region (region 3: nt 284–297; 5′CACCCACUGCCACC3′) in the CR4/CR5 domain (*42*) (Fig. 6A). We also chose a region (region c: nt 141–156; 5′GCCGCCUUCCACCGUUC3′) in the pseudoknot domain of the 5′-half domain as a negative control (Fig. 1).

To confirm that the selected candidates are actually the binding sites of **R17**, we carried out competition experiments using DNA fragments (**cD2**, **cD3** and **cDc**) that contain sequences complementary to the selected regions 2, 3 and c, respectively. The DNA competitors were designed so that their sequence contain complete matches for the regions and form some extra base pairs outside the regions to facilitate binding (**cD2**: d-CGGGGTTCGGGGGCTGGG); **cD3**: d-GCGGTGGCAGT GGGTGCC; **cDc**: d-TGAACGGTGGAAGGCGGCA).

Competition mobility-shift assay was performed on **R17**-hTRW and **R17**-hTR(207–451) complexes with addition of increasing amount of the competitors (1–100-fold excess with respect to hTR derivatives) (Fig. 5). The results for hTRW and the 3′-half molecule were very similar suggesting again that major **R17** binding sites are in the 3′-half domain. No competition was observed for **cDc**. In the case of **cD2**, strong competition was observed even at a **cD2**/hTR ratio of 1:1 but the thin band of R17-hTR complex remained even at a **cD2**/hTR ratio of 100:1. This result suggests that specific competition occur at region 2 and that there may be another binding site in the 3′-half domain. In the case of **cD3**, competition was clearly observed at **cD3**/hTR ratio of 100:1. This result may suggest stronger binding of **R17** to this region, making it more resistant to replacement by **cD3**. However this explanation does not seem compatible with the result for **cD2** that breaks most of the **R17**-hTR complexes at much lower concentrations. A plausible explanation for this inconsistency may be that **R17** binds to both regions 2 and 3, but region 2 is a weaker binding site for **R17**, and competitor binding to one region simultaneously affects **R17** binding to the other region.

Design of hTR Variants, in which Region 1 can Interacts with Region 2 or Region 3—To examine the effects of interactions between region 1 and the other two regions, we designed hTR variants with mutations in these regions enabling them to interact with each other. At first, a sequence of region 1 that does not interact with any other regions of hTR was designed. The sequence of region 1 was modified mainly reducing consecutive G stretches by replacing appropriate G with mostly A or U and adjusting the other bases if necessary. By using ClustalW, the candidate sequences were screened to select a sequence that does not contain any long-matched segment with hTRW. The selected sequence from over one hundred candidate sequences was 5′GGAAAGUAUAGAGUGUC3′ (1M sequence) and an RNA fragment containing this sequence (**R1M**) was prepared. Next, hTR variants containing the 1M sequence and its complementary sequence in region 2 (2M sequence) or region 3 (3M sequence) were designed. In the case of hTR variants containing the 2M sequences (nt 229–242: 5′GACACUCUAUAUUU3′),

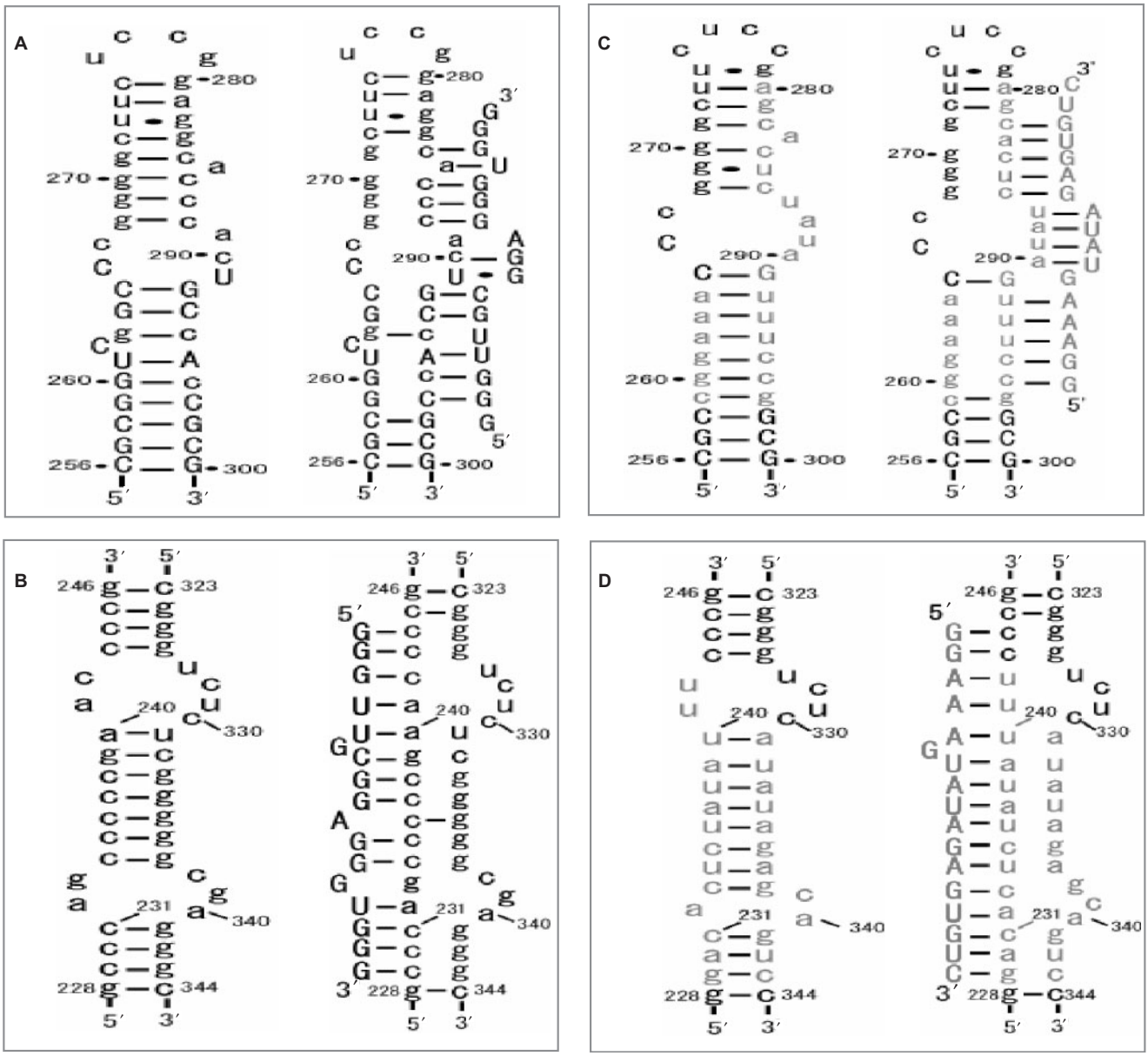

Fig. 6. **Sequences and possible secondary structures of regions 2 (B and D) and 3 (A and C) of hTRW (B and A), hTR2M (D) and hTR3M (C).** The putative associated forms of the regions with **R17** (B and A) or **R1M** (D and C) fragments are also presented in the right side of each box. The sequences of 2M and 3M mutations are written with gray letters. The bases conserved by more than 80% among vertebrates are written with capital letters. The secondary structures for the regions 2 and 3 in hTRW are those proposed by Chen *et al*. (*11*) and Theimer et al. (*42*), respectively. The secondary structures for the hTR variants were derived by calculation using RNAstructure (*43*).

the opposite strand sequence (nt 331–343) was also replaced with a corresponding sequence (5′AUAUAGAGCAGUC3′) to maintain the proposed secondary structure in this region (Fig. 6D) (*11*). In the case of hTR variants containing the 3M sequences (nt 280–297: 5′UCUAUAGUUUCCG3′), the opposite strand sequence (nt 259–264) was also replaced with a corresponding sequence (5′CGGAAA3′) for the same reason (Fig. 6C) (*42*). The secondary structures for the regions 2 and 3 shown in Figures 6C and 6D were obtained by calculation using RNAstructure (http://rna.urmc.rochester.edu/rnastructure.html) (*43*). It was also taken into consideration that **R1M** can bind to the target sequences (2M and 3M) in a similar manner to that for **R17** binding to the putative binding sites (Fig. 6). Thus we prepared seven hTR variants containing mutations in one to three regions of hTR: hTR1M, hTR2M, hTR3M, hTR1M2M, hTR1M3M, hTR2M3M and hTR1M2M3M.

Binding Experiments for R17 and R1M to the hTR Variants—Binding affinity of **R17** and **R1M** to hTRW and its variants were examined by gel mobility shift analysis using ^{32}P-labelled RNA fragments with

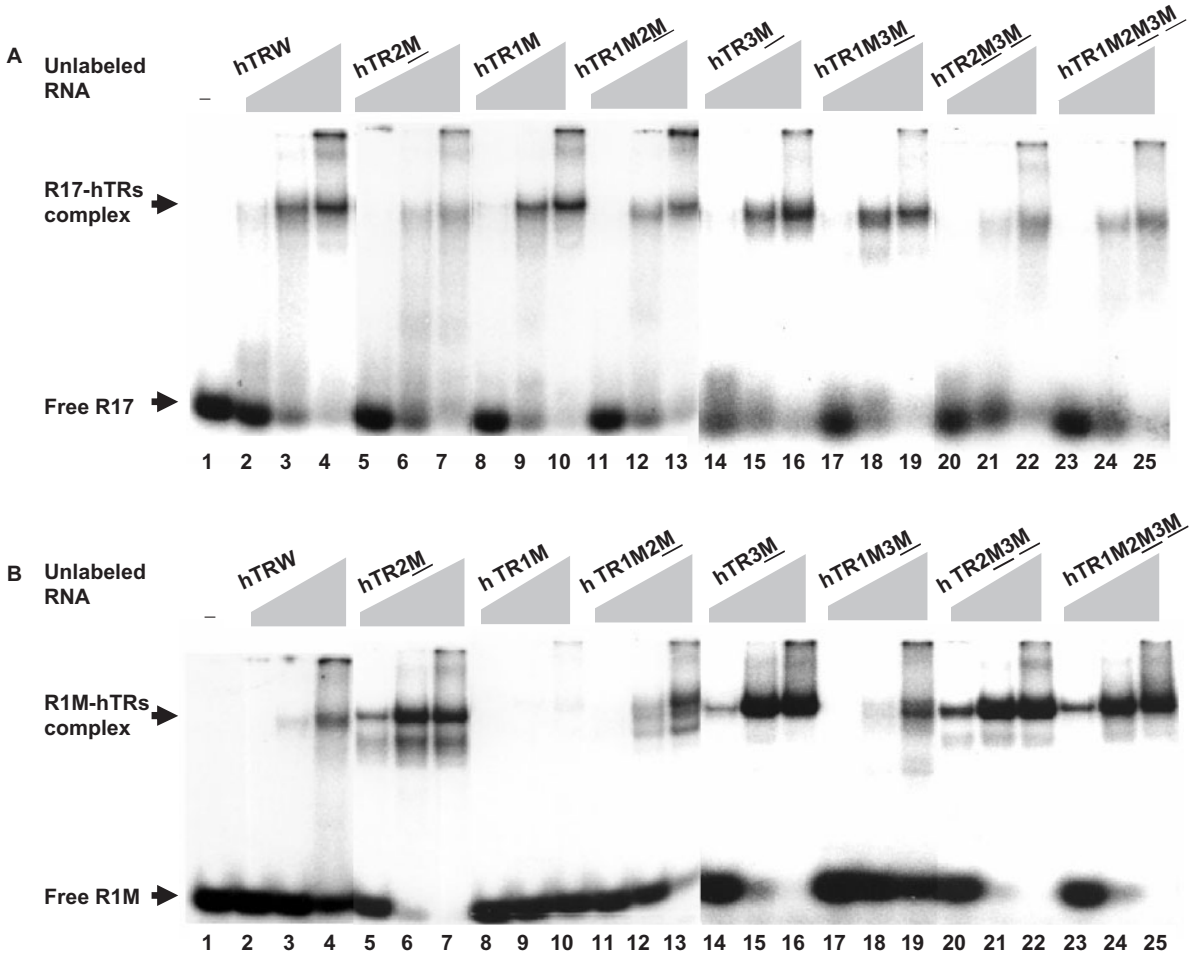

Fig. 7. **Binding experiments for R17 (A) and R1M (B) to hTRW (lanes 1–4) and the hTR variants (lanes 5–25).** Increasing amounts of hTRs (10–1000 nM) were added to ^{32}P-labelled **R17** or **R1M** and the mixtures were analysed by the gel mobility shift assay. Concentrations of hTRs are 10, 100 and 1000 nM in the left lane, middle lane and right lane for each hTR, respectively.

increasing hTR concentration (10–1000 nM) (Fig. 7). In the case of **R17** binding, hTR1M and hTR3M showed almost the same results (Fig. 7A, lanes 8–10 and lanes 14–16, respectively) as that for hTRW (Fig. 7A, lanes 2–4) while hTR2M showed lighter smear bands (Fig. 7A, lanes 5–7) suggesting weaker and/or non-specific binding. These results may imply that **R17** has higher affinity for region 2 than that for region 3. When the results for hTR2M3M (Fig. 7A, lanes 20–22) and hTR1M2M3M (Fig. 7A, lanes 23–25) are compared with that for hTRW, the lighter smear bands observed for the variants suggest weaker binding of **R17** to them than that for hTRW due to the loss of the complementary sequences.

In the case of **R1M** binding, hTR1M showed almost no binding (Fig. 7B, lanes 8–10) while hTRW showed much weaker binding (Fig. 7B, lanes 2–4) when compared with the result for **R17**-hTRW. hTR variants containing 2M and/or 3M mutations (hTR2M, hTR3M, hTR2M3M and hTR1M2M3M) showed very strong binding even at 100 nM while hTR1M2M and hTR1M3M showed definitely weaker binding when compared with the former variants. In the case of the former variants, they have one or two strong binding-sites for **R1M** allowing strong binding. In the case of the latter variants, each variant has a 1M sequence and one complementary target sequence (2M or 3M), therefore, intramolecular interactions between these sequences may reduce accessibility of **R1M** to the variant. These results clearly reveal that the variants have properties as expected from the design.

Effects of Intramolecular Interactions on Telomerase Activity—Effects of the designed mutations in regions 1–3 on telomerase activity were examined (Fig. 8). hTR1M and hTR2M showed much higher activity than hTRW (Fig. 8A, lanes 3, 2 and 1, respectively). hTR1M2M showed even higher activity (Fig. 8A, lane 4). The higher activity of hTR1M may be due to the lack of unfavourable intramolecular interaction involving region 1. The higher activity of hTR2M may be due to another effect. Moreover, hTR1Δ2M, in which nt 1–20 are deleted, also showed higher activity than that for hTRΔ20 (Fig. 8B, lanes 5 and 1). This result suggests that the 2M mutation itself enhances telomerase activity.

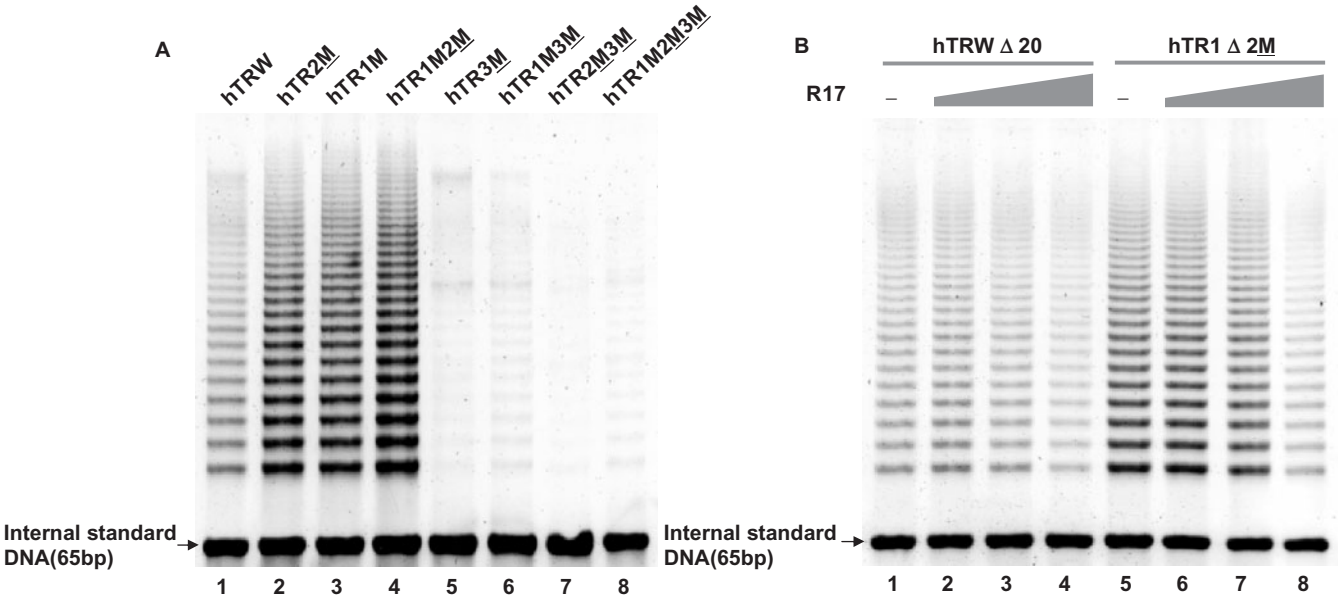

Fig. 8. **(A) Telomerase activity of hTRW (lane 1) and hTR variants containing one to three mutations in regions 1–3 (lanes 2–8).** (B) Effects of **R17** addition on telomerase activity of hTRΔ20 (lanes 1–4) and hTR1Δ2M that contains deletion of nt 1–20 and the 2M mutation (lanes 5–8). **R17** was added to each hTR (200 ng) at **R17**/hTR ratios: 0 (lanes 1 and 5); 10 (lanes 2 and 6); 50 (lanes 3 and 7); 100 (lanes 4 and 8). All the primer extension reactions were carried out with added K^+ ions (150 mM).

It is rather surprising that the 2M mutation itself in the hypervariable paired region enhances telomerase activity. We examined the secondary structure of hTRW and its variants by calculation using RNAstructure (*43*). We found that hTR2M and hTR1Δ2M gave the same secondary structure as proposed from the phylogenetic and conformational analyses (*11*, *15*, *22*, *42*) for the CR4/CR5 domain and the hypervariable region (Fig. 6C and 6D). The most significant point is that the structure contains the P6.1 helix, which is adjacent to region 3 (Fig. 1) and is very important for interaction with hTERT and telomerase activity (*15*, *22*). However, hTRW and hTRΔ20 did not give a secondary structure including the P6.1 helix but gave a structure, in which the four conserved base-pairs (nt 243–246:nt 323–326) just above the upper internal loop (Fig. 6D) and the P6.1 helix are broken and rearranged to form two new hairpins with some bulges. The difference may be mainly due to the difference in the sequences of the internal loop (-U241-U242- for hTR2M vs. -A241-C242- for hTRW). The latter sequence may enable hTRW to form a new hairpin with a bulge at A252 in the region (nt 242–254) causing rearrangement in the other region including P6.1 helix (nt 302–314). These results suggest that hTR2M has a more stable conformation that is favourable for telomerase activity and the favourable conformation is relatively unstable for hTRW. This difference may explain the enhanced activity for hTR2M and hTR1Δ2M.

The result for hTR1M2M showing the highest activity among the three variants suggests that intramolecular interactions between the regions 1 and 2 do not repress telomerase activity but rather enhance the activity. The result also suggests that the intramolecular interactions between regions 1 and 2 may be favourable for proper folding of hTR molecule, where the template region in the core domain and the P6.1 region in the CR4/CR5 domain are in close proximity (*29*).

In sharp contrast to the above results, all hTR variants containing the 3M mutation (hTR3M, hTR1M3M, hTR2M3M and hTR1M2M3M) showed markedly reduced telomerase activity when compared with that for hTRW (Fig. 8A, lanes 5–8). These results show that the 3M mutation itself is unfavourable for telomerase activity and that some base sequence element in this region is important for the activity. It is reported that the sequence and structure of an asymmetric internal loop (-C266-C267- and -A289-C290-U291-) in this region is important for both interaction with hTERT and telomerase activity (*9*, *21*, *22*). The 3M mutation contains modifications in the latter loop component (Fig. 6C).

To elucidate possible repressive effects of interactions with region 1, the effects of **R17** addition to hTR variants with deletion in region 1 (hTRΔ20 and hTR1Δ2M) on telomerase activity were examined (Fig. 8B). For both variants, the telomerase activity decreased with increasing amount of **R17** (1–100-fold with respect to hTR) (Fig. 8B, lanes. 2–4 and 6–8). These results suggest that **R17** binding to the variants represses the telomerase activity. It is assumed that the binding site is not region 2 but possibly region 3.

Effects of Mutations in Region 1 on Telomerase Activity—To elucidate size and sequence requirement for the repressive effect of region 1, some variants with deletion and sequence replacement in region 1 of hTRW were prepared and their telomerase activity was examined (Fig. 9). The deletion variants newly prepared (hTRΔ7 and hTRΔ14) contain deletion of the first 7 and 14 nt in region 1, respectively. The telomerase

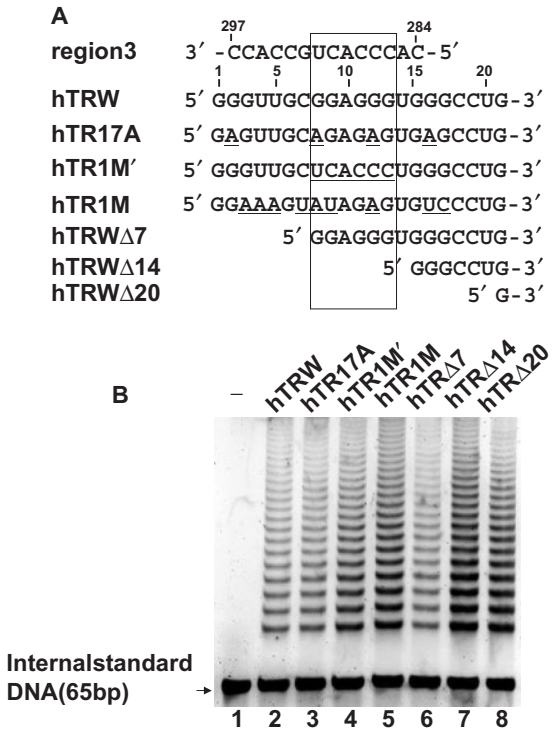

Fig. 9. **(A) Sequences of region 3 in hTRW and of region 1 in the hTR variants.** The positions of nt 8–13 are boxed. (B) Telomerase activity of hTR variants containing sequence mutations (lanes 3–5) or deletion mutations (lanes 6–8) in region 1. Lanes 1 and 2 shows reactions without hTR and with hTRW, respectively. All the primer extension reactions were carried out with added K^+ ions (150 mM). When the telomerase activity was quantitated by the first band and normalized to the internal standard and that of hTRW (100%), hTR17A, hTR1M′, hTRΔ7, hTRΔ14 and hTRΔ20 showed the average activity of 88%, 139%, 122%, 283% and 287%, respectively, with the errors of 3–45%.

activities for the three deletion variants were compared with that for hTRW (Fig. 9B, lanes 6–8 and 2). The hTRΔ7 variant showed about the same activity as that for hTRW but hTRΔ14 and hTRΔ20 showed markedly enhanced activity. These results suggest that the sequence element between nt 8–14 is mainly responsible for the repressive effect of region 1. This segment (nt 8–14: 5′-GGAGGGU-3′) corresponds to the opposite segment (nt 285–291: 5′-ACCCACU-3′) of the putative duplex formed between the regions 1 and 3. The latter segment contains the ACU sequence, which is a component of the asymmetric internal loop and is important for binding to hTERT and telomerase activity (*9*, *21*, *22*). hTRW and hTRΔ7 contain the sequence which can cover the loop sequence but hTRΔ14 and hTRΔ20 do not. hTR17A also contains such a sequence capable of covering the same target.

To confirm the above possibility, a sequence variant (hTR1M′), in which the sequence of nt 8–13 of hTRW (5′-GGAGGG-3′) is replaced with 5′-UCACCC-3′ to break the putative association in the loop region, was prepared and its telomerase activity was examined. hTR1M′ showed enhanced activity with respect to that for hTRW and hTR17A (Fig. 9B, lane 4). hTR1M, in which the sequence has fewer matched segments especially in the region downstream of the ACU loop, showed even higher activity (Fig. 9B, lane 5). These results are again consistent with the idea that the repressive effect is due to the interactions between the regions 1 and 3.

Intramolecular interactions between regions 1 and 3 are possible since the close proximity of the template region and P6.1 region has been shown (*29*). The high telomerase activity for hTR1M2M also supports the idea. The proximity of the two regions makes intramolecular interactions between regions 1 and 3 much easier than intermolecular interactions between **R17** and region 3. The high telomerase activity for hTR2M found in this study will contribute to understanding the dynamic conformational state of hTR. The enhanced activity observed for hTR1M implies that intramolecular interactions within hTR itself can give a mechanism for controlling telomerase activity.

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