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The 5′-End of hTERT mRNA Is a Good Target for Hammerhead Ribozyme to Suppress Telomerase Activity

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Because the expression level of hTERT, a catalytic subunit of human telomerase, is a rate-limiting determinant of telomerase activity, hTERT mRNA would be an excellent target of hammerhead ribozymes for the regulation of telomerase activity. We studied the efficiency of several hammerhead ribozymes targeting hTERT mRNA by transient and stable transfection procedures. To screen the potency of the ribozymes, transient ribozyme transfection and telomerase determination were performed. The ribozyme targeting 13 nucleotides downstream from the 5′-end of hTERT mRNA (13-ribozyme) exhibited the strongest telomerase-inhibitory activity, and the ribozyme to target 59 nucleotides upstream from the poly(A) tail showed clear activity. A stable transfection study confirmed that the 13-ribozyme suppressed telomerase. These observations suggest that the 13-ribozyme can regulate telomerase activity and may possess potential for cancer therapy. © 2000 Academic Press

Key Words: **hammerhead ribozyme; endometrial carcinoma; transient transfection; catalytic subunit of telomerase; RT–PCR.**

Telomerase is a ribonucleoprotein enzyme that maintains the protective structures at the ends of eukaryotic chromosomes, called telomeres (1). In most human somatic cells, telomerase expression is repressed, and telomeres shorten progressively with each cell division (2). In contrast, most human tumors express telomerase, resulting in stabilized telomere length (3). These observations indicate that telomere maintenance is essential to the proliferation of tumor cells and that telomerase is an important target for the development of anti-neoplastic therapies.

Telomerase is composed of an RNA molecule and proteins (4). The RNA component of human telomerase (hTR) functions as a template for the extension reaction of the telomere repeat. It is expressed even in normal tissues, and the expression level is not proportional to the telomerase activity (5). To date, two proteins were identified as the protein components of human telomerase. Telomerase-associated protein 1 (TEP-1) interacts with hTR (6). It has been demonstrated that the expression level is not proportional to the telomerase activity of tissues and cells (7). Human telomerase reverse transcriptase (hTERT), the other protein component of the telomerase, is a catalytic subunit of telomerase, and it has been repeatedly shown that its expression level parallels telomerase activity (8, 9). In addition, *in vitro* reconstitution studies have shown that the essential components of telomerase are the hTR and hTERT (10, 11). These observations indicate that hTERT is the best molecule for telomerase targeting anticancer strategy.

Hammerhead ribozymes are catalytic RNA molecules. They are being increasingly considered and utilized as human gene therapeutic agents for human malignancies (12, 13). These ribozymes utilized as gene therapeutic agents are, in most cases, trans-acting hammerhead ribozymes based on the model of Haseloff and Gerlach (14). The ribozymes efficiently catalyze the phosphodiester bond following GUC GUA, and GUU sequences (15). Due to the broad distribution of such triplet sequences in RNAs, the ribozymes can target most RNA molecules. However, it is very difficult to predict the potency of the ribozymes, because a number of factors are involved in the action of the ribozymes. To obtain a very potent ribozyme, the construction of a ribozyme vector and gene transduction studies are usually performed. We report here that screening of the activity of the ribozymes by a ribozyme transfection procedure resulted in the discovery of a potent ribozyme to suppress telomerase activity in endometrial carcinoma cells.

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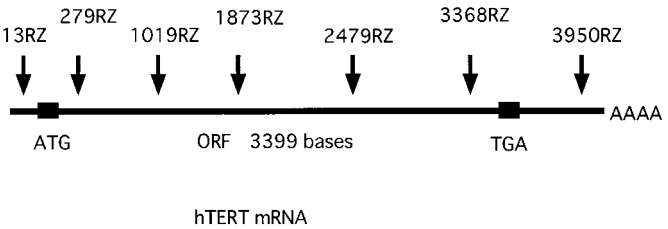

FIG. 1. Target sites of the designed ribozymes. Seven ribozymes of 44 nucleotides long were designed to target different sites of hTERT mRNA. The target sites of the 179-ribozyme to the 3368-ribozyme are inside the open reading frame of hTERT mRNA.

MATERIALS AND METHODS

Cell culture. Ishikawa and AN3CA cells, endometrial carcinoma cell lines, were used in this study. The cells were maintained in Eagle's MEM supplemented with 10% fetal bovine serum (FBS) under an atmosphere of 95% air/5% CO_2 at 37°C.

Ribozyme design and synthesis. The hTERT mRNA sequence was published as hEST2/hTRT (8, 9), and the sequence of the 5′-untranslated region of hTERT mRNA was revised by Horikawa and Takakura (16, 17). Because trans-acting hammerhead ribozymes preferentially recognize and cleave the GUC sequence, we studied the GUC sequence as possible cleavage sites. Within hTERT mRNA of approximately 4.0 kb long, there are 57 GUC-sequences. Considering the complementarity between the upstream and downstream sequences from each GUC, we chose seven target sites (from the 5′-end of hTERT, 11–13, 277–279, 1017–1019, 1871–1873, 2477–2479, 3366–3368, 3948–3950). The target sites of the ribozyme are shown in Fig. 1. The ribozymes were named after the cleavage site from the 5′-end of the RNA (13-ribozyme, 279-ribozyme, 1019-ribozyme, 1873-ribozyme, 2479-ribozyme, 3368-ribozyme, and 3950-ribozyme, respectively). All the ribozymes are comprised of a catalytic core sequence and the franking 10 nucleotides antisense sequences. The structure of the 13-ribozyme and the target region of hTERT mRNA are shown in Fig. 2.

To produce the ribozymes, the bacteriophage T7 RNA polymerase system was used. A set of oligomers was designed to make the DNA template. One primer contains a T7 RNA polymerase promoter sequence followed by the 5′-half of the ribozyme sequence; the other primer contains the anti-sense sequence of the ribozyme. Seventeen oligonucleotides from the 3′-end of both primers were complimentary to each other. The sequences of the oligonucleotides are shown in Table 1.

The primers were mixed to form a hemiduplex, and a PCR amplification of 25 cycles was performed under the conditions of 94°C, 1 min; 40°C, 1 min; and 72°C, 1 min. Unincorporated dNTP was eliminated with a Sephadex G25 Quick Spin Column (Boehringer Mannheim Biochemica, Inc., Tokyo, Japan). The transcription of RNA from the synthetic DNA template was carried out using a T7-MEGAshortscript kit (Ambion, Inc., Austin, TX). The transcription reaction mixture contained 200 ng template DNA, 40 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 30 μM NTP, 1 U/μl recombinant ribonuclease inhibitor, and 1.0 U/μl T7 RNA polymerase in a 20-μl volume. The reaction was carried out at 37°C for 2 h. The reaction mixture was treated with RNase-free DNase, followed by phenol-chloroform extraction and ammonium acetate ethanol precipitation.

Introduction of ribozymes into Ishikawa cells. Ishikawa cells (5×10^4) were seeded in a 6-well plate and incubated for 2 days. The medium was replaced with serum-free Dulbecco's modified MEM (DMEM). Ribozymes were synthesized using a T7-MEGAshortscript kit (Ambion, Inc.) as described above.

Fifteen μg of the ribozyme was mixed with 30 μl *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP, Boehringer Mannheim, Inc., Tokyo) in a total volume of 75 μl of Hepes buffer (20 mM, pH 7.4) and incubated for 15 min at room temperature. The mixture was suspended in 2.0 ml of DMEM medium. Cells were exposed to the ribozyme/DOTAP mixture every 12 h.

Cells were harvested at 48 h after the first exposure to the ribozyme and submitted to telomerase detection assay.

Construction of ribozyme expression vector and transfection. Two single-stranded oligodeoxynucleotides were synthesized such that the 44-base pair ribozyme contained the flanking *Sal*I and *Hin*dIII restriction sites on both ends: 13-ribozyme, tcgagcgcagcagctgatgagtccgtgaggacgaaacgcagcgcagctgcgctgcgtttcgtcctcacggactcatcagctgctgcgc; 3950-ribozyme, tcgaccccaatttctgatgagtccgtgaggacgaaacccacaggagctcctgtgggtttcgtcctcacggactcatcagaaattgggg. They were 5′-phosphorylated by T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA), annealed and cloned into the pHβAPr-1-neo (18). The constructed vectors were designated as pHβAPr-1-neo-13RZ or pHβAPr-1-neo-3950RZ, respectively.

Lipofection of Ishikawa cells with these plasmid vectors was done according to the protocol which the manufacturer(GIBCO-BRL) recommended. As a vector control, transfection with pHβAPr-1-neo was performed In brief, approximately 5×10^4 cells were transfected with 10 μg of the vector DNA that had been complexed with 50 μl of lipofectin (GIBCO-BRL, Rockville, MD). Three days after the transfection, G418 was added to the medium to a final concentration of 1 mg/ml. The transfected cells were exposed to G418 for 4 weeks.

Quantitative RT–PCR for hTERT expressions and ribozyme expression. Total RNA was extracted from the transfectants and parental Ishikawa cells using Isogene (Nippongene, Inc., Tokyo, Japan). One microgram of total RNA from each transfectant was reverse-transcribed with a random hexamer to generate the first-strand cDNA mix. Each cDNA mix was equally divided into three parts, in which each served as a template for an independent PCR.

For hTERT mRNA and beta-actin, primer sets (ggatgaagcggagtcttgga and cggaagagtgtctggagcaa, and ggacctgactgactacctaatgaa and tgatccacatctgctggaaggtgg, respectively) were designed. PCR products were analyzed by electrophoresis on 2.0% agarose gel.

To detect expression of the ribozyme, a set of primers (5′-AGCACAGAGCCTCGCCTTT from the β-actin 5′-untranslated region and 5′-TGGATCCCTCGAAGCTT from a plasmid polylinker) was designed on the basis of the sequence of pHβAPr-1-neo plasmid. The RT–PCR was performed and PCR products were further analyzed by Southern blot with a ^{32}P-labeled probe derived from the ribozyme catalytic core sequence (5′-CCTCACGGACTCATCAG).

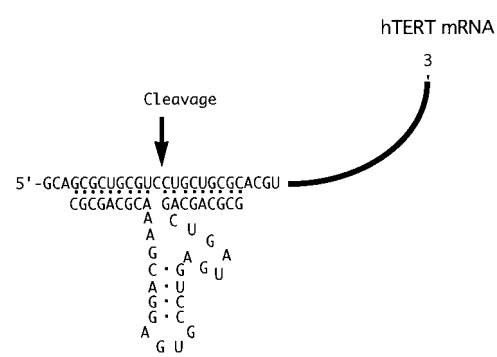

FIG. 2. Structure of 13-ribozyme. The 13-ribozyme is designed to recognize from 4 nucleotides downstream from the 5′-end of hTERT mRNA through the 22-nucleotide and to cleave between the 13th and the 14th nucleotide.

TABLE 1

Primer Sets for the Ribozyme Synthesis

13-ribozyme	5′GGATCCTAATACGACTCACTATAGGGCGCAGCAGCTGAT3′
	5′CAGCGTGGTTTCGTCCTCACGGACTCATCAGCTGCTGCG3′
279-ribozyme	5′GGATCCTAATACGACTCACTATAGGTTCAGGCAGCTGAT3′
	5′GCCAGGTGTTTCGTCCTCACGGACTCATCAGCTGCCTGA3′
1019-ribozyme	5′GGATCCTAATACGACTCACTATAGGACACCGGGGCTGAT3′
	5′ACGCCTTGTTTCGTCCTCACGGACTCATCAGCCCCGGTG3′
1873-ribozyme	5′GGATCCTAATACGACTCACTATAGGATGCTGCCTCTGAT3′
	5′AGCAGAGGTTTCGTCCTCACGGACTCATCAGAGGCAGCA3′
2479-ribozyme	5′GGATCCTAATACGACTCACTATAGGGCGTAGGAACTGAT3′
	5′CTTCGACGTTTCGTCCTCACGGACTCATCAGTTCCTACG3′
3368-ribozyme	5′GGATCCTAATACGACTCACTATAGGGGAGCTTCCCTGAT3′
	5′CAGCTGAGTTTCGTCCTCACGGACTCATCAGGGAAGCTC3′
3950-ribozyme	5′GGATCCTAATACGACTCACTATAGGCCCCAATTTCTGAT3′
	5′CCTGTGGGTTTCGTCCTCACGGACTCATCAGAAATTGGG3′

Telomerase detection assay. Cultured cells were washed once with phosphate-buffered saline and were scraped into a buffer (10 mM Hepes–KOH, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM dithiothreitol). The cells were washed in the buffer and then homogenized in 200 μl of a cell lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (Chaps, WAKO Chemical Industries, Inc., Osaka, Japan), and 10% glycerol) and incubated on ice for 30 min. Cell homogenates were then centrifuged at 12,000*g* for 20 min at 4°C. The supernatant was recovered, and snap-frozen in liquid nitrogen and stored at −80°C. The concentration of protein was measured with protein assay dye (Bio-Rad Laboratories, Hercules, CA).

The TRAP assay was performed using a TRAP*EZE* Telomerase detection kit (Oncor, Inc., Gaithersburg, MD). In brief, 2 μl of tissue extract and 48 μl of TRAP reaction mixture consisting of the 5′-end-labeled TS primer (5′-AATCCGTCGAGCAGAGTT) with [γ-^{32}P]ATP, 50 μM of dNTP mix, a TRAP primer mix (RP primer, K1 primer, and TSK1 template) and 2 IU *Taq* DNA polymerase in 20 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.05% Tween 20, and 0.01% BSA were mixed and incubated at 30°C for 30 min. PCR was then performed as follows: 94°C, 30 s; 60°C, 30 s; 25 cycles. PCR products were electrophoresed on 12% acrylamide gel and autoradiographed.

RESULTS AND DISCUSSION

The ribozymes that we used in the transient transfection study were 44 nucleotides long, and such a length of RNA can be synthesized in large quantities at a very low cost *in vitro.* For the screening of potency of the ribozymes, direct ribozyme transfection would be more advantageous than that of a ribozyme vector, because the construction of the ribozyme vector requires much labor and expense. First, we studied the *in vivo* activity of the ribozymes by this method. Fifteen micrograms of ribozymes was administered to Ishikawa and AN3CA cells every 12 h. The 13-ribozyme showed the highest inhibitory activity, and the 3950-ribozyme, which targeted 59 nucleotides upstream from the poly(A) tail, showed clear activity in both cell lines (Fig. 3). No other ribozymes showed any telomerase inhibitory activity. Both sites are located near the end of the hTERT mRNA, so it is likely that they had escaped the self-folding.

Next, we constructed two kinds of expression vectors. The 13-ribozyme or 3950-ribozyme sequence was subcloned into the *Sal*I and *Hin*dIII sites of the pHβAPr-1-neo vector. The constructed vectors were introduced into Ishikawa cells. Tens of clones resistant to G418 were obtained from each transfectant. In 10 arbitrarily chosen clones and a pooled clone, the telomerase activity was screened. The 13-ribozyme reduced telomerase activity in most clones (Fig. 4), whereas in the 3950-ribozyme transfectant, no clones exhibited any reduced telomerase activity (data not shown).

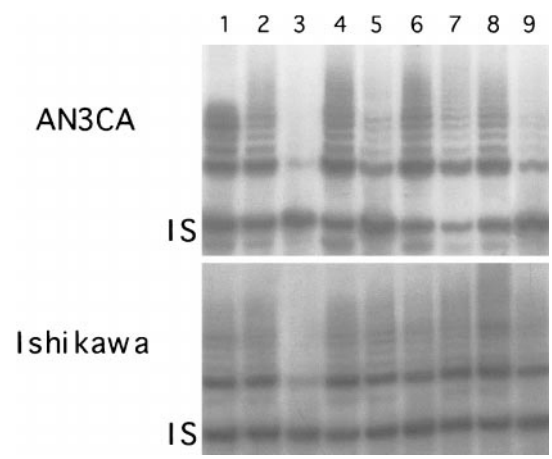

FIG. 3. Telomerase activity by transient transfection of the ribozymes. 15 μg of ribozymes was administered with DOTAP, a cationic liposome. The telomerase activity was determined at 48 h. Upper AN3CA cells, lower Ishikawa cells. Lane 1, parent cells; lane 2, liposome only; lane 3, 13-ribozyme; lane 4, 279-ribozyme; lane 5, 1019-ribozyme; lane 6, 1873-ribozyme; lane 7, 2479-ribozyme; lane 8, 3368-ribozyme; lane 9, 3950-ribozyme. Note that the 13-ribozyme exhibits the strongest telomerase-inhibitory activity in both cell lines. In the 3950-ribozyme, obvious suppression of telomerase is observed.

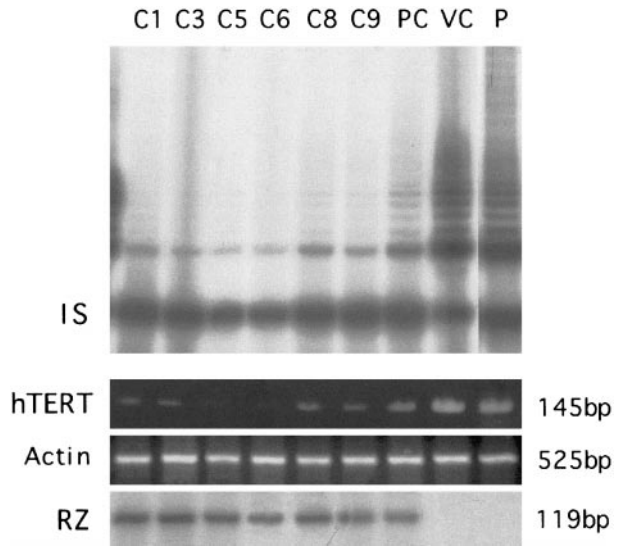

FIG. 4. Stable transfection of 13-ribozyme. The ribozyme sequences were subcloned into pHβAPr-1-neo and introduced into Ishikawa cells. In arbitrarily chosen G418-resistant clones, the telomerase activity was determined (upper) and the hTERT mRNA expression was semi-quantified by RT–PCR (middle). The ribozyme expression was analyzed by RT–PCR and Southern blot (lower). Note that telomerase activity is suppressed in the clones in which the 13-ribozyme was introduced. In such clones, a decrease in hTERT mRNA expression and a steady expression of the ribozyme are observed. C1–C9, clones; PC, a pooled clone of 13-ribozyme transfectant; VC, vector control; P, parental Ishikawa cells.

In the clones exhibiting attenuated TA(clones 1, 3, 5, 6, 8, 9, and the pool, the expression of hTERT mRNA was quantified by RT–PCR. The expression level was clearly decreased in the clones showing attenuated telomerase activity. In clones 5 and 6, the signal of the hTERT transcript was faint. The expression level of hTERT mRNA was mostly correlated with telomerase activity. In these clones, the expression of the ribozyme was studied by RT–PCR and subsequent Southern blot analyses. In all cases transfected with pHβAPr-1-neo-13RZ, a steady state expression of the ribozyme was observed (Fig. 4).

Besides catalytic activity, the ribozymes possesses an antisense property. For these antisense sequences of the ribozymes to recognize a target region, a number of factors influence its feasibility (e.g., the accessibility of target site, the specificity of the ribozyme, etc.) (19). The base number for the recognition sequence determines the specificity of the ribozyme, but affects its kinetics (20). We designed a total of 20 antisense nucleotides for target site recognition, and such a length will be sufficient to recognize the target region (21). However, the entire ribozyme sequence including the recognition sequences will have a chance for non-Watson Crick base pairing with other molecules (22). Such base-pairings will reduce the potency of the ribozyme. Furthermore, for the ribozyme to exert catalytic activity, the proper three-dimensional structure of the ribozyme is required (23, 24). In this context, the entire sequence of the 13-ribozyme would be most favorable not only for the formation of the ribozyme's three-dimensional structure, but also for its specificity. Of the pHβAPr-1-neo-13RZ, the subcloned ribozyme was transcribed into an approximately 200-nucleotide RNA from the human beta actin promoter. Of the transcript, only 44 nucleotides were the sequence of the ribozyme. The surplus vector sequence was inevitably included in the transcript. These sequences might affect the specificity as well as the cleavage activity of the ribozyme. Thus, the discordance of the 3950-ribozyme efficiency between the transient and stable transfection studies might be explained by the vector sequence.

A limited number of chemical agents are known to regulate the telomerase activity. Retinoids reduce the telomerase activity of leukemic cells with the induction of cellular differentiation (25). Tamoxifen also reduces the telomerase activity of hormone-responsive breast carcinoma cells with cell growth retardation (26). 9-Hydroxyellipticine exerts telomerase inhibitory activity by inhibition of the protein kinases in pancreatic cancer cells (27). These agents display clear telomerase inhibitory effects, though the effect would be limited in certain cells. Therefore, such special nucleic acids are a hopeful choice for the regulation of the telomerase activity in a broad spectrum of cancer cells. Because hTR and hTERT are essential elements for telomerase activity, blocking of the hTR and/or hTERT mRNA by these nucleic acids is a major strategy.

Regarding hTR, a number of studies have demonstrated that the template region is crucial for the telomerase regulation (28, 29). Therefore, this region is a excellent target for the ribozymes (30) and antisense oligonucleotides (31). The efficient target sites of hTERT mRNA, however, have never been demonstrated. As described before, the hTERT mRNA is more ideal as a target of these molecules than hTR. We first demonstrated the possible target site of hTERT of hammerhead ribozymes, and this will offer hope for ribozyme-mediated anti-telomerase cancer therapy.

We observed significant growth retardation of clones with suppressed telomerase activity. With respect to the clone 6, the cell growth was maintained during the first 2 months after the transfection, and then gradually retarded. The doubling time of clone 6 was 48 h at the 10th week after transfection against 27 h of parental Ishikawa cells. The growth potential was further reduced and then almost disappeared within 3 months. All the cells of clone 6 died within 4 months. We studied the nuclear morphology of the growth-arrested cells and some cells showed condensed chromatin and frag-

mented nuclei. Because these findings were characteristic in cells undergoing apoptosis (32), we considered that the cells died by apoptosis.

It has been reported that telomerase inhibition by antisense oligonucleotide against hTR causes growth inhibition (33, 34) and that exogenous expression of dominant negative mutants of hTERT (35, 36), and peptide nucleic acid and 2′-O-MeRNA oligomers complementary to the template region of hTR (37) induce apoptosis following strong telomerase inhibition. Our observations of the 3-ribozyme were in agreement with those reports, indicating that the 13-ribozyme could be a potential anticancer agent.

With respect to the 13-ribozyme, there is room for improvement of the ribozyme activity, for instance, by altering the length of the recognition sequence. The trials to improve the activity of the 13-ribozyme are progressing

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