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Design of RNA enzymes distinguishing a single base mutation in RNA

Makoto Koizumi, Yoji Hayase, Shigenori Iwai, Hiroyuki Kamiya, Hideo Inoue and Eiko Ohtsuka

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

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ABSTRACT

RNA enzymes (ribozymes) which can cleave RNA by recognizing sequences of 9-15 bases are described. Substrates must contain UX (X=U, C or A). A ribozyme consisting of two oligoribonucleotides (19 mer and 15 mer) was shown to cleave a ribo 11 mer catalytically with Km and k_{cat} values of 0.53 μM and 0.03 min^{-1}, respectively. A non-cleavable substrate-ribozyme complex containing 2'-O-methylnucleoside was prepared and CD spectra were compared at different temperature. In order to obtain an efficient ribozyme, a one-strand RNA with a chain length of 37 was prepared. The ribozyme was shown to distinguish a single base mutation in mRNA's which were prepared by transcription of two synthetic DNA duplexes coding for positions 7-26 of c-Ha-*ras* protein. The mutant (Val-12) mRNA which had GUU was cleaved but the wild type mRNA which contained GGU was not changed, when treated by the ribozymes in the presence of Mg^{2+}

INTRODUCTION

Self-cleaving RNA and self-splicing RNA have been found in a variety of biological systems. These RNA's are divided into 4 categories: 1) Self-splicing RNA found in group I introns of the rRNA precursor of *Tetrahymena*[1]; 2) Group II introns in yeast mitochondrial mRNA which form a lariat[2]; 3) RNA component in RNase P which cleaves tRNA precursors at the 5'-site[3]; 4) Self-splicing RNA mainly found as infectious RNA for plants e.g. a satellite RNA of tobacco ringspot virus (sTobRV)[4,5] avocado sunbloch viroid[6], a virusoid of lucerne transient streak virus (vLTSV)[7], and transcripts of satellite 2 DNA of the newt[8], which cleave specifically in the presence of magnesium. These satellite RNAs have been assumed to form a "hammerhead" secondary structure[6] for the self-cleavage domain. Although a mechanism for participation of magnesium ions has not been elucidated, formation of the 2',3'-cyclic phosphate and 5'-hydroxyl groups

has been identified, as found with Pb(II)-catalyzed cleavage of the yeast $tRNA^{phe}$ [9].

Transcripts from short DNA using T7 RNA polymerase have proved that the consensus sequence and these stemmed structures are important for cleavage[10,11]. We have also reported the chemical synthesis of 21 mers for the construction of a "hammerhead" and made alterations in the consensus sequence[12]. From the results of experiments using those 21 mers, ribozymes were designed to cleave specific sites of RNA such as 5S rRNA[13]. In this paper we report the catalytic properties of a two-strand ribozyme and the design of a more efficient ribozyme to distinguish a point mutation. Temperature dependent properties of a non-cleavable RNA complex are also described.

MATERIALS AND METHODS

Oligonucleotides

Oligoribonucleotides were synthesized by the phosphoramidite method using 2'-O-tetrahydropyranyl and 5'-O-dimethoxytrityl protecting groups as described previously[12]. An oligoribonucleotide containing 2'-O-methylcytidine was prepared using 4-N-benzoyl-5'-dimethoxytrityl-2'-O-methylcytidine 3'-(N,N-diisopropyl)methoxyphosphoramidite[14,15] as a condensing unit in a mechanical synthesis as above by a DNA synthesizer, Applied Biosystems 380A. Deblocked oligonucleotides were purified by reversed phase and ion-exchange chromatography.

Oligodeoxyribonucleotides were also synthesized by the procedure similar to the above materials using the phosphoramidite derivatives of deoxyribonucleosides[16].

Preparation of ribo 72 mers by transcription of the DNA template

The DNA template was prepared by joining 7 DNA fragments with T4 DNA ligase as described for the synthesis of genes for peptides[17]. The template (1 μM) in 30 μl of 40 mM Tris-HCl (pH 8.1), 20 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 0.01% (v/v) Triton X-100, 0.05 μg/μl BSA, 4 mM 4NTP and 8% (w/v) polyethylene glycol 6000 was treated with 28 U/μl T7 RNA polymerase (Pharmacia) at 37°C for 2 hr[18]. The mixture was shaken with phenol-chloroform and the product was desalted by passing though a column of Sephadex G-50 in 0.1 M triethylammonium bicarbonate. The single

strand RNA (72 mer) was purified by 10% PAGE in the presence of 8M urea.

5'-Labelling of short oligonucleotides and ribo 72 mers

Oligoribonucleotides (100 pmol) were labelled with [γ-^{32}P]ATP(New England Nuclear) and 1U of T4 polynucleotide kinase (Takara Shuzo) in 5 μl of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The 5'-phosphorylated products were isolated by using NENSORB 20 (Dupont).

The ribo 72 mer (25 pmol) in 0.1 M Tris-HCl (pH 8.0) was incubated with 0.22 U of bacterial alkaline phosphatase (Takara Shuzo) at 37°C for 1 hr, and treated with phenol-chloroform twice. The polynucleotides were labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and desalted with NENSORB 20.

Preparation of a one-strand ribozyme (37 mer)

Oligoribonucleotide (UACCCUGAAACAGCGCG, 5 nmol) was treated with 0.67 mM ATP and 10U of T4 polynucleotide kinase at 37°C for 1 hr, and was shaken with phenol-chloroform. The 5'-phosphorylated product was desalted with a column of Sephadex G-25 in 0.1 M triethylammonium bicarbonate.

The 5'-phosphorylated oligoribonucleotide (pUACCCUGAAACAGCGCG) dissolved in 20 μl of H_2O was incubated with 2.5 μl of 0.1 M $NaIO_4$ at 0 °C for 70 min in the dark, and 50 μl of 0.1 M ethylene glycol was added to stop the reaction at 0 °C for 30 min in the dark. The mixture was treated with 50 μl of 2 M lysine-HCl (pH 8.2) at 45 °C for 2 hr, and desalted with Sephadex G-25. The 5', 3'-diphosphorylated product (pUACCCUGAAACAGCGCp) was purified by anion-exchange HPLC.

Two oligoribonucleotides (acceptor; CUACACCCUGAUGAAGGGUGA and donor; pUACCCUGAAACAGCGCp) in 50 μl of 50 mM HEPES-NaOH (pH 7.5), 20 mM $MgCl_2$, 100 μM ATP, 3.3 mM DTT, 0.001% BSA and 10% DMSO were incubated with 1.4 U/μl T4 RNA ligase (Takara Shuzo) at 6 °C for 13.5 hr, and treated with phenol-chloroform. The linked product was purified by anion-exchange HPLC.

Cleavage reaction in the presence of Mg^{2+}

Oligonucleotides were incubated in 25 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5) and 20 mM NaCl at 15°C. Aliquots were mixed with the loading solution for 20% polyacylamide gel electrophoresis in the presence of 8M urea or homochromatography that was performed

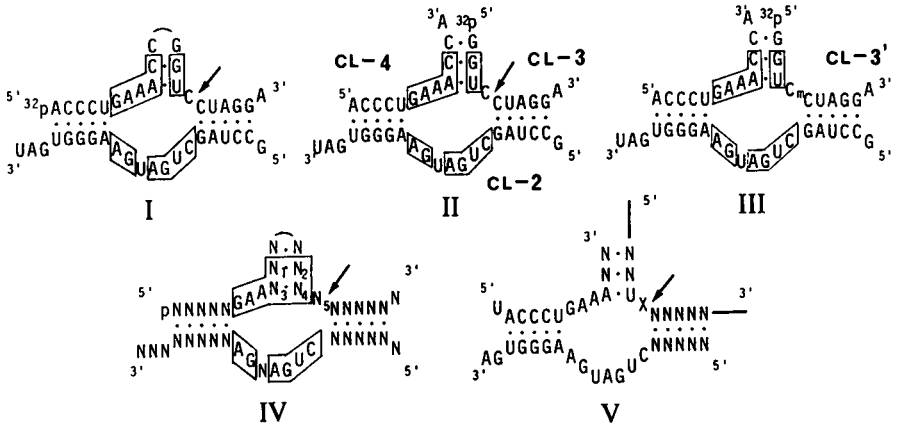

Fig. 1 Self-cleavage RNA and catalytic RNA. Complex I, self-cleaving RNA containing the sequence of the satellite RNA of the newt; complex II, a substrate (CL-3) and catalytic RNA (CL-2 and 4); complex III, non-cleavage substrate (CL-3'); complex IV, exchangeable bases in the consensus domain (N_1•N_2 can be A•U, G•C, U•A or C•G. N_3•N_4 can be A•U and N_5 can be A, C or U.); complex V, substrate and ribozymes (N can be A, G, C or U and X can be A, C or U.)

using Homo-mix III[19]. Cleavage rates were estimated by cutting the gel and counting the radioactivity with a liquid scintillation counter after autoradiography.

Enzymatic digestion

RNase A (Boehringer Mannheim) was used in 100 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Cleavage reaction with RNase T_1 (Sankyo) and RNase PhyM (Pharmacia) were performed in 25 mM sodium citrate (pH 5.0), 0.125 mg/ml tRNA, 1 mM EDTA and 7 M urea.

Measurement of CD spectra

CD spectra were recorded by JASCO J-500A spectropolarimeter. Oligonucleotides (1 A_{260}) were dissolved in a buffer used for cleavage reaction in the presence of Mg^{2+}.

RESULTS AND DISCUSSION

Design of ribozymes for cleavage of RNA at the 3'-side of UA, UC or UU.

From the previous study[12] on the self-cleavage reaction of chemically synthesized 21 mers containing the cleavage domain of a satellite RNA of the newt (complex I in Fig. 1), it became

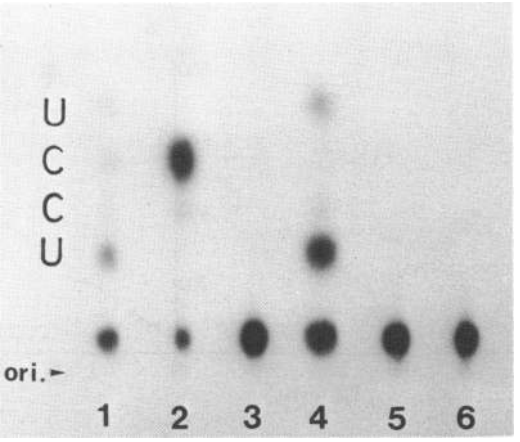

Fig. 2 Homochromatography of cleavage products. Lane 1, marker oligonucleotides, RNase A digestion products of CL-3 in complex II (Fig. 1); lane 2, CL-3 in the presence of 25 mM $MgCl_2$; lane 3 CL-3 in the presence of 5 mM EDTA; lane 4, RNase A digestion products of CL-3' in complex III (Fig. 1); lane 5, CL-3' in the presence of 25 mM $MgCl_2$; lane 6, CL-3' in the presence of 5 mM EDTA.

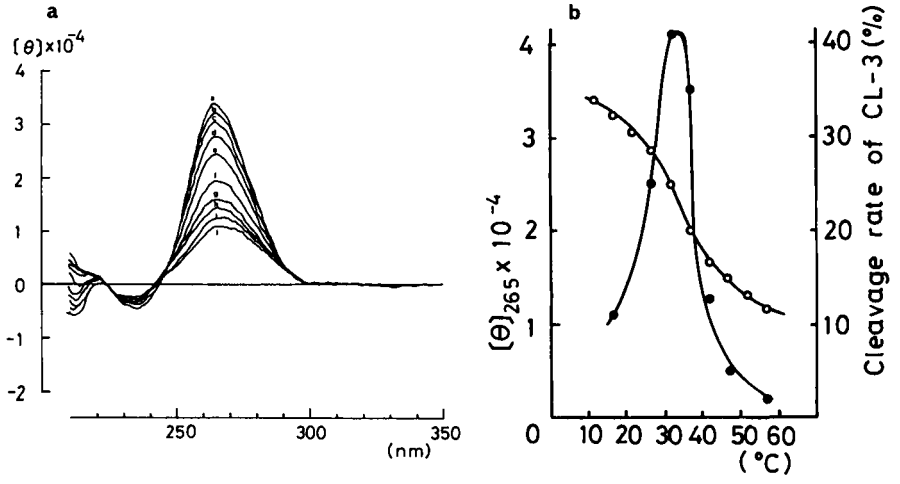

Fig. 3 (a) CD spectra for complex III (Fig. 1). Spectra were measured at a, 12 °C; b, 17 °C; c, 22 °C; d, 27 °C; e, 32 °C; f, 37 °C; g, 42 °C; h, 47 °C; i, 52 °C; j, 57 °C.
(b) Cleavage rates of CL-3 (solid circles) and $[\theta]_{265}$ of complex III (open circles) at different temperatures.

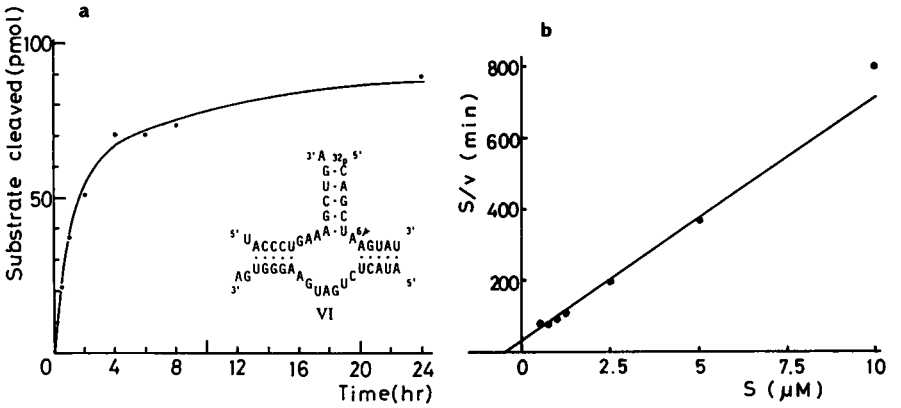

Fig. 4 (a) Catalytic hydrolysis of pCAGCUAAGUAU with the two-strand ribozyme. The substrate (100 pmol) was treated with the ribozyme (20 pmol) in 20 μl of 40 mM Tris-HCl (pH 7.5), 20 mM NaCl and 25mM $MgCl_2$ at 15 °C. Aliquots (2.5 μl) were taken at different time intervals and mixed with 50 mM EDTA. Ratios of cleaved products were analyzed by 20% PEGE / 8M urea and counted by a scintillation counter. (b) Hanes-Woolf plot of the cleavage reaction shown in Fig. 4a. The substrate (10-500 pmol) was treated with 10 pmol of the ribozyme in the solution described above.

possible to design a ribozyme (CL-2 and 4 in complex II in Fig. 1) for cleavage of a substrate (CL-3). The results of the experiments for cleavage of CL-3 shown in Fig. 2 in the presence and absence of Mg^{2+} indicated that the cleavage occurred as expected. Complex III was prepared to confirm participation of the 2'-hydroxyl group in cleavage. Introduction of 2'-O-methylcytidine instead of cytidine at the cleavage position prevented the reaction, as shown in Fig. 2 (lane 5). This does not conflict with the previous analysis[12] of the 3'-end of cleaved products, and the availability of non-cleavable complexes containing the consensus bases for self-cleavage is very useful for structural studies of metal ion dependent cleavage of RNA. CD spectra for complex III are reported in the next section. Complex IV summarizes exchangeable (N_1•N_2) and non-exchangeable (N_3•N_4=A•U) bases in the consensus domain. Application of this information to design ribozymes for the sequence dependent hydrolysis of RNA afford a more generalized scheme as shown in

Fig. 5 (a) Preparation of an RNA containing the valine codon GUU from a synthetic DNA template with T7 RNA polymerase. (b) Preparation of a one-strand ribozyme using T4 RNA ligase and synthetic RNA.

complex V where X can be A, C or U. Thus two oligonucleotides (e.g. 19 mer and 12 mer) can be used as restriction ribozymes which recognize sequences, $(N)_nUX(N)_n$ (X=A, C or U).

CD spectra for a non-cleavable complex

CD spectra for the non-cleavable substrate (CL-3') which contains 2'-O-methyl groups at the cleavage site are shown in Fig. 3a. $[\theta]_{265}$ values at the peak changed mostly at 32-37°C are shown in Fig. 3b. Certain changes in structure may have occurred below and above the temperature. The initial cleavage percentages of complex II (Fig. 1) were plotted at the same temperature (Fig. 3b) and the maximum rate was obtained near 32°C. Probably a tertiary structure of complex II which was necessary for cleavage collapsed above 30°C resulting in loss of efficiency of cleavage. A three-stemmed structure stabilized by hydrogen bonds may be important for cleavage and sizes of stems should be considered in designing ribozymes consisting of two

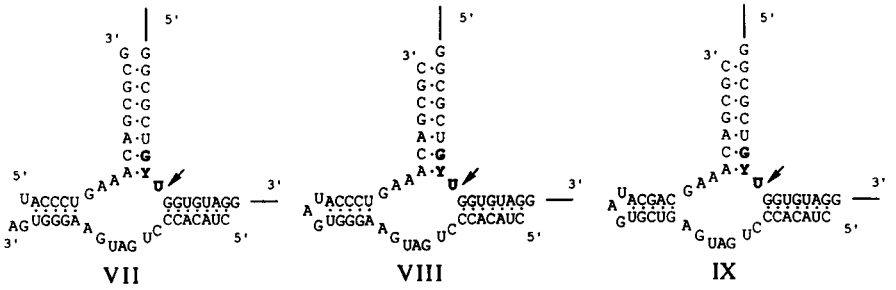

Fig. 6 Secondary structures of one-strand and two-strand ribozymes with c-Ha-ras mRNA. Y=G represents wild type RNA and Y=U represents mutant RNA.

oligoribonucleotides. In fact, shorter stemmed complexes have shown less efficient cleavage[13].

Kinetics of cleavage of a 11 mer with two oligoribonucleotides.

The catalytic nature of a ribozyme consisting of a 19 mer and 15 mer designed for the cleavage of a 11 mer which contained a 5'-splice site[13] was investigated as shown in Fig. 4a using a 5 fold excess of the substrate (11 mer). After 2 hr, 51% of the substrate was cleaved and the reaction proceeded to 90% after 24 hr. Then, kinetic parameters were obtained by the Hanes-Woolf plot using 10 pmoles of the ribozyme and 10-500 pmoles of the 11 mer (Fig. 4b). The Km, Vmax and k_{cat} values were found to be 0.53 μM, 0.015 $\mu M \cdot min^{-1}$ and 0.03 min^{-1}, respectively. These results are similar to those of Uhlenbeck's[11] and Jeffries'[20] systems.

Preparation and reaction of one-strand ribozymes to distinguish a point mutation.

As described above, the 3'-side of UX (X=A, C or U) can be cleaved by a ribozyme. Since a single base change (G to T) in the c-Ha-ras gene at position 12 has been known to result in Gly to Val mutation[21,22], mRNA's from those two genes were considered to be ideal model substrates for a ribozyme. Two mRNAs were prepared by transcription of synthetic DNA duplexes containing the T7 promoter[18]. Fig. 5a shows a scheme for preparation of an RNA containing GUU at position 12. The other strand was obtained by the same procedure except using a template coding for GGU instead of GUU. Two partially-paired oligoribonucleotides (21 mer and 17

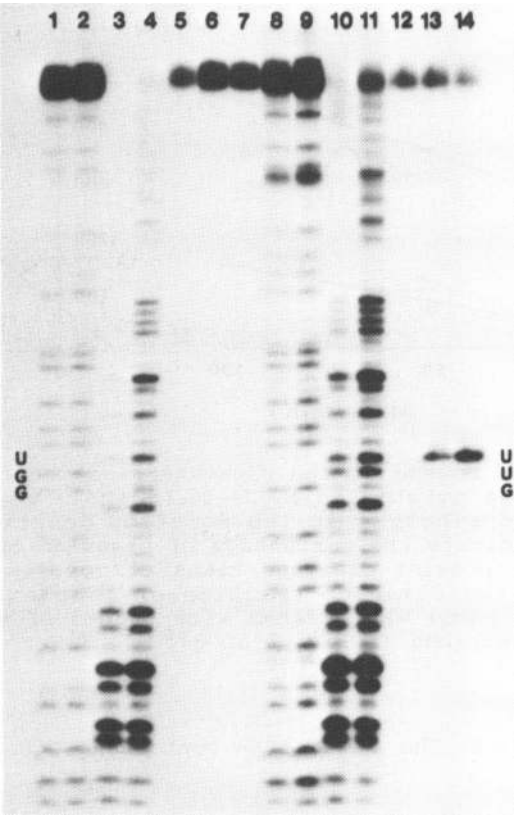

Fig. 7 Autoradiogram of the cleavage products of complex VIII(Fig. 6). 5'-labelled ribo 72 mer (6.25 pmol) was treated with one-strand ribozyme (7.5 pmol) in 4.6 µl of 40 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$ and 20 mM NaCl at 37 °C, and was mixed with loading solution for 10% PAGE/8M urea. In lanes 1-7, the substrate was wild type RNA (GGU). In lanes 8-14, the substrate was mutant RNA (GUU). Lanes 1 and 8, RNase T_1 (0.5 U) digestion; lanes 2 and 9, RNase T_1 (0.1 U) digestion; lanes 3 and 10, RNase PhyM (5 U) digestion; lanes 4 and 11, RNase PhyM (1 U) digestion; lanes 5 and 12, no incubation; lanes 6 and 13, for 4 hr; lanes 7 and 14, for 23hr.

mer) were designed as a ribozyme and chemically synthesized. Complex VII (Fig. 6) shows a possible secondary structure of the two-strand ribozyme and a mRNA containing the valine codon (GUU). The 5'-side of the cleavage site can form a Watson-Crick type

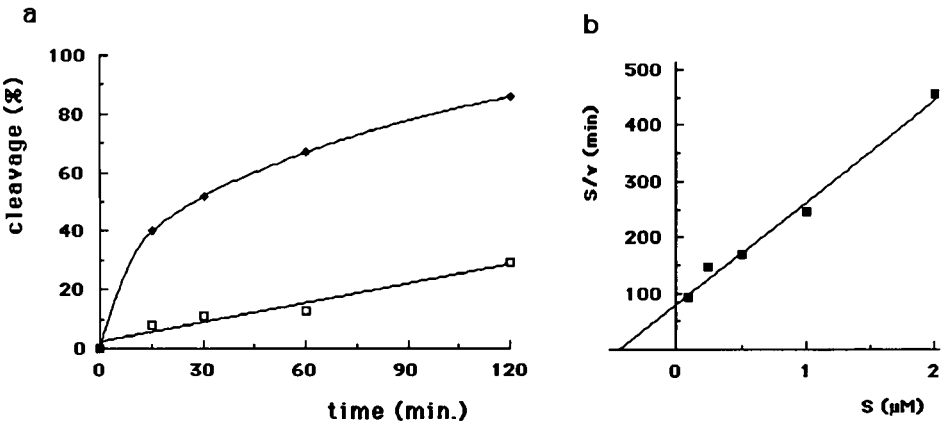

Fig. 8 (a) Time course of the cleavage reaction of complex VIII and IX (Y=U). The mutant RNA (GUU) (6.25 pmol) was treated with 7.5 pmol of the ribozyme in the solution described in Fig. 7. Open squares indicate the percentage of cleavage of complex VIII. Solid squares represent cleavage rates of complex IX. (b) Hanes-Woolf plot of the cleavage reaction of complex IX (Y=U). The substrate (1-20 pmol) was treated with 5 pmol of the ribozyme in the solution described in Fig. 7 at 37°C.

hydrogen bond, A•U. The other mRNA containing GGU contains an A•G mismatch.

Cleavage reactions of these complexes in the presence of Mg^{2+} at 37°C showed 17% reaction with the strand containing the valine codon GUU after 23 hr and the wild type RNA (GGU) was not changed. The result was as expected. However, the rate of the reaction was slower than predicted. In order to increase the efficiency of the cleavage reaction, the two strands of the ribozyme were joined by T4 RNA ligase[23] as shown in Fig. 5b. The extent of cleavage of the joined ribozyme (complex VIII in Fig. 6) increased to 61% after 23 hr as shown in lane 14 in Fig. 7. The wild type mRNA was not cleaved (lane 7 in Fig. 7). The cleavage site was confirmed by digestion with RNase T_1 and RNase PhyM. However, an unfavorable hairpin structure, which was formed by $^{5'}$CACCCU$^{3'}$ and $^{5'}$AGGGUG$^{3'}$ of the ribozyme in complex VIII, was identified and assumed to be a possible reason for this slow reaction. Another ribozyme was prepared by reforming sequences such that a hairpin structure could not be formed. The complex of

newly prepared ribozyme with the mRNA is shown in Fig. 6 (complex IX) and a comparison of the time course using these two ribozymes is shown in Fig. 8a. The cleavage reaction of complex IX was ca. 20-times faster than that of complex VIII. It is shown that the stability of secondary structure in the ribozyme is important for the cleavage of targeted RNA. Then, a Hanes-Woolf plot, which was used to determine the kinetic parameters, showed that the ribozyme of complex IX cleaved the mutant RNA catalytically (Fig. 8b). The Km, Vmax and k_{cat} values were found to be 0.44 μM, 0.0055 $\mu M \cdot min^{-1}$ and 0.011 min^{-1}, respectively. The catalytic activity of complex IX could be compared with that of complex VI, which had a 11 mer as the substrate. The k_{cat}/Km value of complex IX was one half that of complex VI. Because the substrate of complex IX, which was 72 nucleotides, probably forms a complex tertiary structure, the cleavage reaction of complex IX was slower than that of complex IV.

RNases, exhibiting base specific cleavage, have been found, and used as probes for determining RNA sequence and secondary structure[24]. However, no sequence-specific RNA endonuclease's have been found. Recently, procedures for sequence-dependent cleavage of RNA have been reported. An altered form of the Tetrahymena IVS RNA can act as an endoribonuclease[25]. Chimeric oligonucleotides and RNase H have been used to cleave a complimentary RNA strand[14,26]. Hybrid enzymes that had hydrolytic activity have been prepared by covalently linking oligonucleotides[27,28]. Haseloff and Gerlach reported that RNA enzymes could cleave transcripts of the chloramphenicol acetyl transferase gene, but those ribozymes were designed for recognizing a GCU sequence[29]. We have designed synthetic ribozymes which can cleave specific sites of RNA containing $(N)_nUX(N)_n$ (X=A, C or U). The ribozymes described in this paper can distinguish between RNAs differing in only a single base mutation.

Ribozymes hybridize to the target RNA in a similar way to that of antisense RNA[30,31] used for interference with expression of genes. However, the RNA enzymes act catalytically as an endonuclease, and thus they may inhibit expression of various genes and viruses more dramatically than antisense RNA.

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