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Sequence-specific cleavage of RNA by designed ribozymes

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Ribozymes that distinguish a single base change in RNA were synthesized and used to specifically cleave c-Ha-ras messenger RNA. Using phosphorothioate containing oligonucleotide substrates, we have shown that Mg^{2+} binds to the pro-*R* oxygen of the phosphate and that the RNA cleavage reaction occurs via an in-line mechanism. Oligoribonucleotides containing a modified nucleoside are described.

INTRODUCTION

Restriction endonucleases strictly recognize a base sequence in a DNA duplex and cleave the DNA in a sequence-dependent manner.[1] Although base-specific ribonucleases have been found, enzymes that cleave RNA in a sequence-specific manner have not been available. Self-cleaving RNAs from various organisms have been found to catalyse the transesterification or hydrolysis of RNAs, and some catalyse the sequence-specific cleavage of exogenous oligonucleotide substrates.[2] Self-splicing RNAs, which are mostly found as RNAs infectious to plants, such as the satellite RNA of tobacco ring spot virus,[3] the avocado sunblotch viroid,[4] and the transcripts of satellite 2 DNA of the newt[5] are known to have a rather small catalytic domain. These self-splicing RNAs are assumed to form a 'hammerhead' secondary structure. A small domain has been shown to self-cleave in the presence of divalent cations.[6] We have used site-directed mutagenesis of two short oligoribonucleotides to investigate several essential bases in a hammerhead domain for their roles in the cleavage reaction.[7] We found that the catalytic domain can be divided into a two-stranded ribozyme and a substrate RNA.[8] A hammerhead ribozyme derived from the sequence of a satellite RNA has been reported to cleave a GUC site.[9] We have deduced the recognition sequences UA, UU, and UC for specific cleavage sites and have used these ribozymes to distinguish a single base change, G to U, in c-Ha-ras mRNA.[10] The binding site for Mg^{2+} ions and the cleavage mechanism have been determined using decaribonucleotides containing phosporothioates at the cleavage site.[11]

In order to understand these reactions, chemically modified oligoribonucleotides will be useful in structure studies of hammerhead ribozymes. We describe the synthesis of oligoribonucleotides containing a 2′-*O*-(*o*-nitrobenzyl) nucleoside.

RESULTS AND DISCUSSION

Design and synthesis of ribozymes that cleave the GUU site

Previous studies on the self-cleavage reaction of chemically synthesized 21 mers show that hammerhead ribozymes that cleave UA, UC, or UU can be designed, as shown in Figure 1. Using this strategy, a ribozyme with a chain length of 37 was synthesized by joining chemically synthesized ribooligonucleotides. Figure 2 shows the ribozyme substrate on mRNA from a mutated c-Ha-ras gene that contains a single mutation

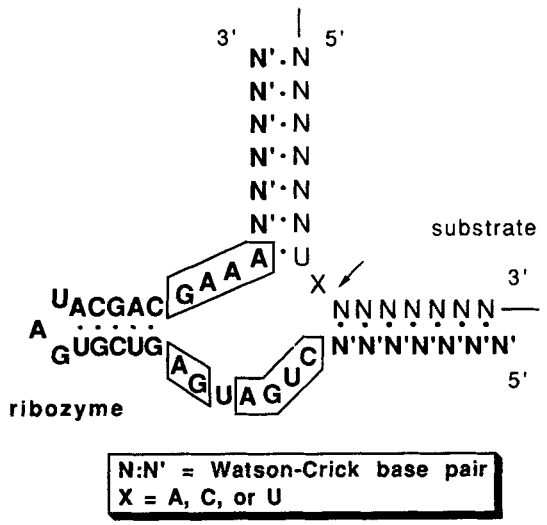

Figure 1 Design of a ribozyme that cleaves UA, UC, or UU.

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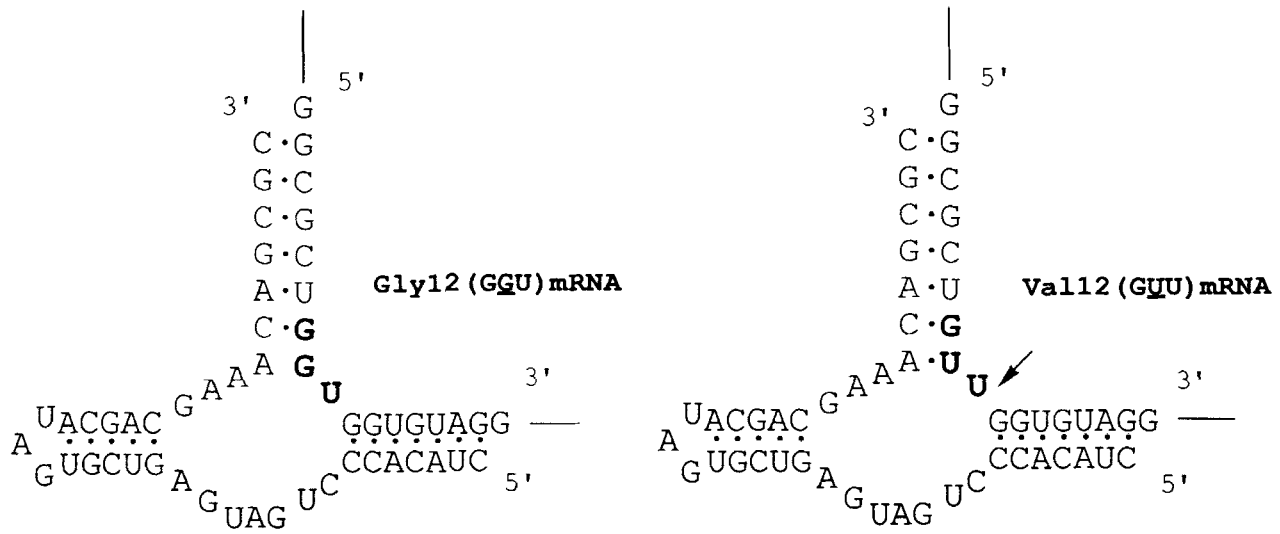

Figure 2 Synthetic ribozyme that recognizes GUU.

at codon 12 (*GUU*). In an *in vitro* reaction, the ribozyme cleaved the 3'-side of GUU in a 5'-labelled ribo 72 mer, which was prepared by transcription by T7 RNA polymerase.[10] The designed ribozyme did not cleave the normal mRNA model containing *GGU* at codon 12.

Mg^{2+} binding site and stereochemical pathway

In order to determine the binding site of Mg^{2+} ions, phosphorothioates were introduced at the cleavage site of a substrate with a chain length of 10.[11] The decaribonucleotide isomers were synthesized by the phosphoramidite method[12] using 2'-*O*-(tetrahydrofuranyl) and 5'-*O*-(dimethoxytrityl) protecting groups, as described previously,[7] except for the oxidation of the phosphorothioate.[13] In this step, the S_8 in pyridine-carbon disulphide triethylamine was used instead of the I_2 in water-pyridine.[13] The completely deblocked isomers were separated by reverse-phase high performance liquid chromatography (HPLC). The hydrolysis rates of these diastereoisomers were compared by using a ribozyme. The *R*p isomer was found to be cleaved more slowly than the wild type. On the other hand, the cleavage rate of the *S*p isomer was faster than that of the wild type. The results indicate that Mg^{2+} binds to the pro-*R* oxygen of the phosphate. A similar result was reported using a hammerhead ribozyme with a different sequence.[14] Using the *S*p substrate, we have analysed the cleavage produce by hydrolysis to the monomeric cyclic phosphorothioate. The cyclic 2',3'-cyclic phosphorothioate was identified by HPLC as having the exo-configuration. This indicates that the cleavage proceeds via the in-line pathway, because the bovine pancreatic RNase, a catalysed transesterification of cytidine 3'-*O*-phosphorothioate methyl ester, with the *S*p configuration, is known to give the exo-compound.[15]

Synthesis of ribozymes containing modified nucleotides for structure studies

We have synthesized substrate oligoribonucleotides containing a 2'-*O*-methylnucleoside at the cleavage site. These non-cleavable substrates were used to construct complexes with ribozymes, which were analysed by circular dichroism in the presence of magnesium ions.[11] These ribozymes are suitable materials for crystallization and X-ray analysis.

As alternative substrates for X-ray studies, we synthesized oligoribonucleotides containing 2'-*O*-(*o*-nitrobenzyl) cytidine[16] at the cleavage site. The modified nucleoside was protected by 5'-*O*-dimethoxytritylation and *N*-benzoylation. The modified oligoribonucleotides were synthesized by the phosphoramidite method using a DNA synthesizer,[17] and were deblocked by the usual method, except for the removal of the 2'-*O*-(t-butyldimethylsilyl) protecting group (Fig 3). At the final deblocking step, the product was subjected to acidic conditions (pH 2) for 35–50 h, and the completely deprotected oligonucleotide was purified by HPLC. The oligonucleotide containing the modified cytidine was not cleaved by the ribozyme and can be converted to the normal substrate by treatment with UV (> 280 nm), because *o*-nitrobenzyl ether is removed by photochemical conversion to *o*-nitrosobenzaldehyde.[18] The converted substrate was susceptible to cleavage in the standard conditions of the ribozyme reaction.

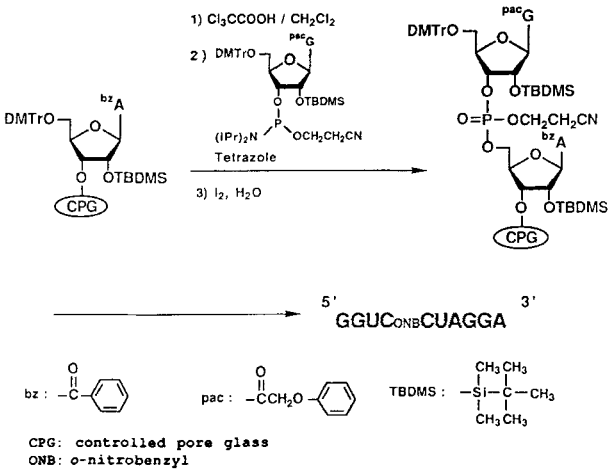

Figure 3 Scheme for synthesis of a decaribonucleotide containing 2′-*O*-(*o*-nitrobenzyl) cytidine.

EXPERIMENTAL SECTION

General methods

Synthesis and purification of oligoribonucleotides were performed as described.[7,11] 2′-*O*-Methylnucleosides were synthesized as described.[19] Cleavage reactions and other general methods were described previously.[10,11] Enzymatic joining and transcription experiments were performed as described.[10]

Protection of 2′-*O*-(*o*-nitrobenzyl)cytidine

2′-*O*-(*o*-Nitrobenzyl)cytidine (1 g, 2.41 mmol) was dried by co-evaporation with pyridine, and was treated with benzoyl chloride (1.11 ml, 2.41 mmol) in pyridine (4.82 ml) plus triethylamine (0.335 ml, 2.41 mmol) for 43 min at 0°C. Water (20 ml) was added to the mixture and the product was extracted with methylene dichloride, and then washed with saturated sodium bicarbonate. The solution was concentrated and the residue was dissolved in ethanol (12 ml) and pyridine (7.2 ml). The mixture was treated with 2 N sodium hydroxide (14.5 ml) for 50 min at 0°C, and was neutralized with 2 N hydrochloric acid. The product was extracted with methylene dichloride and was recrystallized from ethanol. The yield was 736 mg (1.53 mmol, 63%). The 2′-*O*-(*o*-nitrobenzyl)-*N*-benzoylcytidine (710 mg, 1.47 mmol) thus obtained was treated with dimethoxytrityl chloride (598 mg, 1.76 mmol) in pyridine (5.1 ml) for 2 h at room temperature. Methanol (2 ml) was added to the mixture and the product was extracted with methylene dichloride. It was then washed twice with saturated sodium bicarbonate and once with water. The mixture was concentrated and co-evaporated with toluene, and applied to a column of silica gel (C-300). The product was separated by elution with 0.5% methanol in methylene dichloride and was precipitated from the methylene dichloride by *n*-hexane (5 ml). The yield was 861 mg (1.1 mmol, 75%).

Synthesis of a decaribonucleotide (GGUCONBCUAGG)

5′-*O*-Dimethoxytrityl-2′-*O*-(*o*-nitrobenzyl)-*N*-benzoylcytidine (200 mg, 0.255 mmol) was dried by co-evaporation with pyridine, and was dissolved in methylene dichloride (1 ml) and diisopropylethylamine (0.184 ml, 1.02 mmol). A syringe was used to combine the mixture with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.0683 ml, 0.306 mmol). The combination and reaction of these components were performed under a N_2 atmosphere. After 1.5 h, ethyl acetate (15 ml) was added to the solution and the organic layer was washed twice with saturated sodium bicarbonate, and twice with saturated sodium chloride. The solution was filtered through 1 ps and was concentrated. The product was purified by chromatography on silica gel (C-300) using ethylacetate *n*-hexane (2:1, v/v) and was dried by evaporation to a syrup. The yield was 185 mg (0.188 mmol, 74%). The oligonucleotide was prepared with the phosphoramidite derivatives of protected ribonucleosides (American Bio Nuclear Inc.) using a DNA synthesizer (Applied Biosystems, 381A). The *o*-nitrobenzyl derivative prepared above (100 mg, 0.101 mmol) was dissolved in acetonitrile (0.8 ml) and was used in the synthesis, starting from 1 μmol of *N*-phenoxyacetylguanosine linked to controlled pore glass. The coupling time was 900 s in each step. The product was released from the support by treatment with concentrated ammonia for 2 h and was concentrated. The residue was dissolved in ethanol which was saturated with ammonia (20%), at 0°C (5 ml) and was heated at 55°C for 16 h. An aliquot was concentrated and the residue was treated with 0.01 N HCl/dioxane (10 ml; 1:1, v/v) for 50 h. The solution was neutralized with 0.1 N ammonium hydroxide. The produce was desalted by gel filtration on a column (1.8 × 46 cm) of Sephadex G-25 and was purified by reverse-phase HPLC on a column (Inertsil ODS-2, 0.46 × 25 cm) using a gradient of acetonitrile (5–50%, 20 min) in 0.1 M triethylammonium acetate (pH 7). The yield was 2.5 A_{260} units (24 nmol, 12%).

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REFERENCES

1 Nathan, D.; Smith, H.O.: *Annu. Rev. Biochem.* **1975**, *44*, 273.
2 (a) Altman, S.: *J. Biol. Chem.* **1990**, *265*, 20053. (b) Cech, T.R.: *Annu. Rev. Biochem.* **1990**, *59*, 543. Simons, R.H.: *Trends Biochem. Sci.* **1989**, *14*, 445.
3 Buzayan, J.M.; Gerlach, W.L.; Bruening, G.: *Nature* **1986**, *323*, 349.
4 Hutchins, C.J.; Rathjen, P.D.; Forster, A.C.; Symons, R.H.: *Nucleic Acids Res.* **1981**, *14*, 3627.
5 Epstein, L.M.; Gall, J.C.: *Cell* **1987**, *48*, 535.
6 Uhlenbeck, O.C.: *Nature* **1987**, *328*, 596.
7 Koizumi, M.; Iwai, S.; Ohtsuka, E.: *FEBS Lett.* **1988**, *228*, 228.
8 Koizumi, M.; Iwai, S.; Ohtsuka, E.: *FEBS Lett.* **1988**, *239*, 285.
9 Haseloff, J.; Gerlach, W.L.: *Nature* **1988**, *334*, 585.
10 Koizumi, M.; Iwai, S.; Ohtsuka, E.: *Nucleic Acids Res.* **1989**, 7059.
11 Koizumi, M.; Ohtsuka, E.: *Biochemistry* **1991**, *30*, 5145.
12 (a) Baron, A.D.; Tang, J.Y.; Caruthers, M.H.: *Nucleic Acids Res.* **1984**, *12*, 405. (b) Shinha, N.D.; Biernat, J.; MacMamus, J.; Köster, H.: *Nucleic Acids Res.* **1984**, *12*, 4539.
13 (a) Ott, J.; Eckstein, F.: *Biochemistry* **1987**, *26*, 8237. (b) Stein, C.A.; Subasinghe, C.; Shinozuka, K.; Cohen, J.S.: *Nucleic Acids. Res.* **1988**, *16*, 3209.
14 Slim, G.; Gait, M.J.: *Nucleic Acids Res.* **1991**, *19*, 1183.
15 Usher, D.A.; Erenrich, E.S.; Eckstein, F.: *Proc. Natl. Acad. Sci. USA* **1972**, *69*, 115.
16 Ohtsuka, E.; Tanaka, S.; Ikehara, M.: *Chem. Pharm. Bull.* **1977**, 949.
17 Wu, T.; Ogilvie, K.K.: *J. Org. Chem.* **1990**, *55*, 4717.
18 (a) Pachornik, A.; Amit, B.; Woodward, R.B.: *J. Amer. Chem. Soc.* **1970**, *92*, 6333. (b) Amit, B.; Zehavi, U.; Pachornik, A.: *Israel J. Chem.* **1974**, *12*, 103.
19 Inoue, H.; Hayase, Y.; Imura, A.; Iwai, S.; Miura, K.; Ohtsuka, E.: *Nucleic Acids Res.* **1987**, *15*, 1631.