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Cleavage of specific sites of RNA by designed ribozymes

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Two ribozymes were designed for site-specific cleavage of RNA. A UA site in an undecaribonucleotide was cleaved by a ribozyme consisting of two partially paired oligoribonucleotides with chain lengths of 19 and 15. The other ribozyme, which consists of a 19-mer and a 13-mer, recognized a UC sequence at positions 42 and 43 of 5 S rRNA.

Synthetic oligoribonucleotide; RNA enzyme; Endonucleolytic activity; RNA sequence recognition

1. INTRODUCTION

A site-specific self-cleavage reaction has been found in satellite RNA of virus [1,2], infectious RNA in plants [3,4] or transcripts of satellite DNA of the newt [5]. These RNAs undergo the cleavage reaction in the presence of magnesium at neutral pH. The cleavage has been found to occur in a region containing the consensus sequences. The self-cleavage domain has been postulated to form a three-stemmed secondary structure. Some 'hammer-head' short RNAs have been prepared using T7 RNA polymerase and the consensus sequences were proved to be important for self-cleavage [6,7]. We have also constructed a series of several self-cleaving RNA duplexes using chemically synthesized 21-mers [8]. On the basis of the results obtained from these experiments, we have designed a ribozyme containing a partially paired oligoribonucleotide duplex which could cleave a specific site (A6) in an undecamer, pCAGCUAAGUAU, having a sequence similar to the 5′-splice sequence [9]. We have also applied this technique to a naturally occurring 5 S rRNA from *Desulfovibrio vulgaris* MK [10]. Here, we wish to report that cleavage occurs as expected.

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2. MATERIALS AND METHODS

Oligoribonucleotides were synthesized by the phosphoramidite method [11] using a DNA synthesizer (Applied Biosystems 380 A) as described [8]. Monomer units were prepared from 5′-dimethoxytrityl 2′-*O*-tetrahydropyranyl-*N*-protected nucleosides. Oligonucleotides were purified by reversed-phase and anion-exchange HPLC.

The 5 S rRNA was extracted from *D. vulgaris* MK. Purification of 5 S rRNA was performed by Sephadex G-100 gel filtration and 10% polyacrylamide gel electrophoresis [10].

5′-labelling was carried out using [γ-^{32}P]ATP plus polynucleotide kinase (Takara Shuzo), and desalted using Nensorb 20 (Dupont).

Cleavage reactions were performed essentially as described previously [5,8]. Reaction mixtures containing 5′-labelled substrate (2.5 pmol), enzyme strands (3.75 pmol), 25 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5) and 20 mM NaCl in a volume of 2.3 μl were incubated at 15°C for 15 h unless otherwise specified. In control experiments without Mg^{2+}, 5 mM EDTA was added. The reaction was stopped by addition of 50 mM EDTA (3 μl) and the mixture analyzed by electrophoresis on 20 or 10% polyacrylamide in 8 M urea. Markers were obtained by base-specific digestion of 5′-labelled substrate with RNase U_2, RNase T_1 (Sankyo) or RNase *B. cereus* (Pharmacia). The percentage of cleavage was estimated by counting radioactivity in gel fragments using a scintillation counter.

3. RESULTS AND DISCUSSION

3.1. *Cleavage of an 11-mer containing a splice sequence*

The structure of the duplex ribozyme, which consists of a 19-mer (AUACUCUGAUGAAGGGUGA) and a 15-mer (UACCCUGAAAGCU-

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GA), is shown together with the 11-mer substrate strand as complex I (fig.1). Complexes II and III are less efficient ribozymes which form a smaller number of hydrogen bonds with either the 5′- or 3′-end of the substrate. Complex IV is an inactive ribozyme containing a mispaired C with C6 of the 11-mer.

The design of these ribozymes is based on the previous observation [8] that the 3′-site of UN (N = A, C and U) in oligoribonucleotides could be cleaved efficiently. The 5′-splice sequence was selected as the substrate and A6 as the secession point.

The cleavage reaction was performed using a 1.5-fold excess of the enzyme strand in the presence of 25 mM magnesium at 15 or 25°C. The product was analyzed by 20% polyacrylamide gel electrophoresis as shown in fig.2. The extents of cleavage are summarized in the upper 6 rows in table 1.

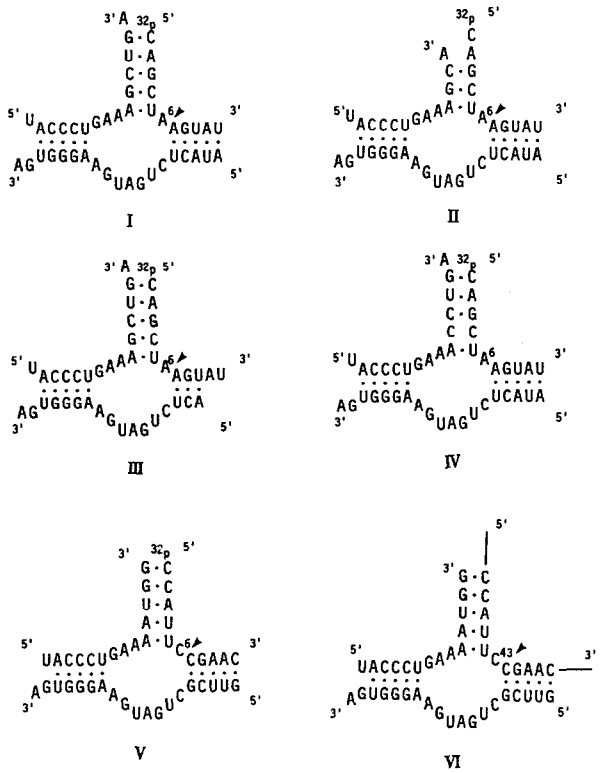

Fig.1. Structures of complexes consisting of a ribozyme and a substrate. The substrates were labelled with radioactive 5′-phosphate. The cleavage sites are indicated by an arrow. Descriptions of complex I–IV are contained in the text.

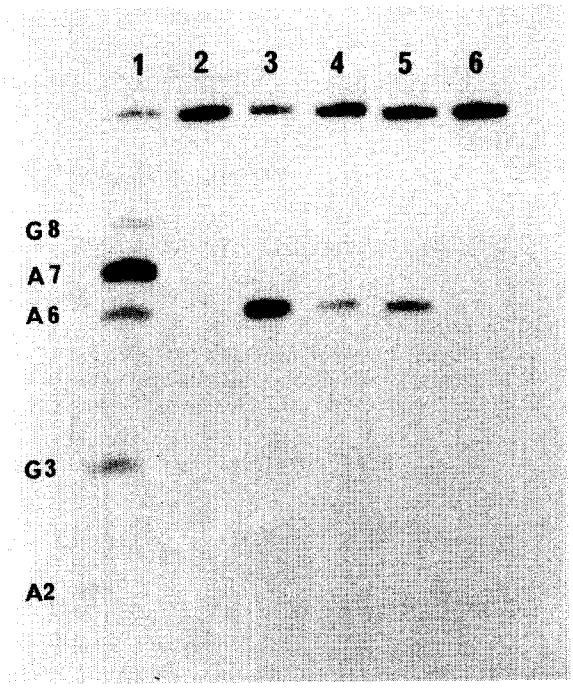

Fig.2. 20% polyacrylamide gel electrophoresis of the cleaved products of complexes I–IV. Lanes: 1, RNase U_2 digestion; 2,3, complex I in the presence of EDTA and Mg^{2+}, respectively; 4, complex II; 5, complex III; 6, complex IV in the presence of Mg^{2+}.

The half-life of the 11-mer in complex I was found to be ~2 h at 15°C. At high temperatures, the reaction seemed to proceed slowly, probably due to disruption of the tertiary structure. Complexes II and III, containing a smaller number of

Table 1

Complex	Ribozyme/ substrate	Temperature (°C)	Time (h)	Percentage of cleavage
I	1.5	15	15	93
		25	15	89
		37	15	8
II	1.5	15	15	22
III	1.5	15	15	28
IV	1.5	15	15	ND
V	1.5	15	15	93
VI	1.5	15	17	1
	10	15	17	6
	40	15	17	9
	100	15	17	20
	200	15	17	33

ND, not detected

hydrogen bonds either at the 3′- or 5′-side, reacted more slowly. Complex IV, containing a mismatch C-C in the consensus sequence of the synthetic ribozyme, did not yield any cleavage product.

It may be concluded from the present experiment that partially paired short oligoribonucleotides can serve as an enzyme, that hybridization of the ribozyme to the substrate is essential for the catalytic reaction, and that the stability of the duplexes is an important factor with regard to the rate of the cleavage reaction.

3.2. *Cleavage of 5 S rRNA by a ribozyme*

We have applied the above observation to site-specific cleavage of 5 S rRNA from *D. vulgaris*. The secondary structure of the 5 S rRNA, which consists of five helices and five loops, is shown in fig.3. Since possible targets for cleavage should contain UN (N = A, C and U), UC (42–43) was selected as a cleavage site. A ribozyme consisting of a 13-mer and a 19-mer was designed and synthesized chemically together with a model substrate which had the same sequence of C38–C48 of the 5 S rRNA. Complexes V and VI in fig.1 show ribozyme-substrate duplexes containing the short substrate and 5 S rRNA, respectively. In complex V, the 11-mer was 93% cleaved in the presence of 25 mM magnesium at 15°C for 15 h and the 5′-labelled 5 S rRNA was cleaved as expected (table 1). Autoradiograms of the cleaved products are shown in fig.4. A weak band one nucleotide longer was observed, because the 2′,3′-cyclic phosphate of the cleaved product

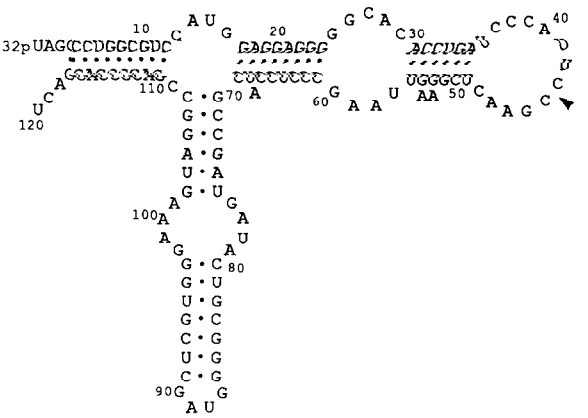

Fig.3. Secondary structure of *D. vulgaris* 5 S rRNA. The cleavage site is indicated by an arrow.

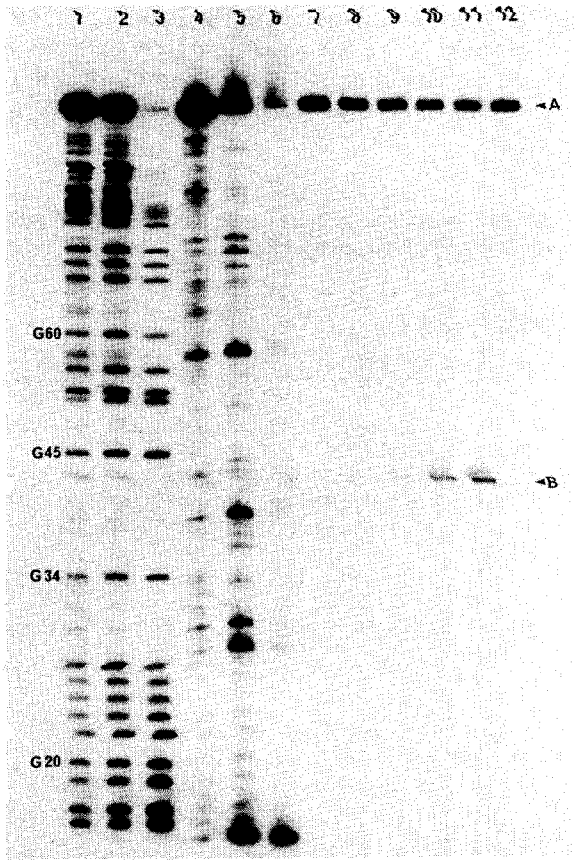

Fig.4. 10% polyacrylamide gel electrophoresis of the cleaved products of complex VI. A and B show 5′-labelled 5 S rRNA and the cleaved product (p43mer), respectively. Lanes: 1–3, RNase T_1 digestion (0.05, 0.1 and 0.5 U, respectively); 4–6, RNase *B. cereus* digestion (0.05, 0.25 and 0.5 U, respectively); 7–11, complex VI (0, 1.5, 10, 100 and 200 equivalents of the enzyme, respectively); 12, complex VI in the presence of 5 mM EDTA.

(p43-mer) was hydrolyzed to the 2′- or 3′-phosphate. The cleavage site (C43) was confirmed by RNase T_1 and RNase *B. cereus* digestions. A 200-fold excess of enzyme strands was found to be necessary for 33% cleavage of the 5 S rRNA, probably due to a loop structure or poor hydrogen bond formation.

A sequence-dependent RNA endonuclease has been prepared by alteration of the intervening sequence of *Tetrahymena* rRNA [12]. Other enzyme-assisted selective hydrolyses of RNA with the complementary modified oligonucleotide [13,14] or a covalently linked oligonucleotide [15] have also been investigated. The present ribozyme can be a

restriction endonuclease which hydrolyzes at the N′ position of RNA containing (N)nUN′(N)n (N = A, G, U and C; N′ = A, U and C). Application of this technique to in vivo experiments is in progress.

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