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In Vitro Selection of Hammerhead Ribozymes with Optimized Stems I and III

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A pool of *cis*-acting hammerhead ribozymes randomized in their substrate recognition sequences was constructed. A variety of active *cis*-acting ribozymes which had various structures of stems I and III was selected from the pool by *in vitro* selection. The selected ribozymes were cloned and sequenced. The relationship between the cleavage efficiency and base-pairing in stems I and III of the selected ribozymes was investigated. The ribozymes with the smaller difference in folding energies between the active conformation and the stable but inactive conformation showed a tendency to have the better cleavage efficiency. The optimum length of stem I was 5 or 6 bases while the longer stem III, in general, appeared to be required for efficient cleavage. The specificity of the ribozyme reaction is discussed in terms of the length of stems I and III.

Keywords: Hammerhead ribozyme, Hepatitis B virus, *In vitro* selection, Substrate recognition sequence

Introduction

Hammerhead ribozymes are catalytic RNAs with sequence specific endonucleolytic cleavage activity (Symons, 1992). The hammerhead RNA self-cleaving domain was originally found in a number of small, circular pathogenic RNAs in plants which replicate via a rolling-circle mechanism (Symons, 1992). The basic features of the hammerhead structure consist of the three base-paired stems I, II, and III, surrounding a single stranded central region, with 15 conserved bases (Fig. 1A) (Uhlenbeck, 1987). Change of any of the conserved bases diminishes catalytic activity, and most conserved bases are involved in RNA folding and catalysis (Tuschl *et al.*, 1995). Thus, the conserved central bases are essential for ribozyme activity. Although there appear to be few restrictions on the nonconserved nucleotides in the three stems, one base-pair in stem III proximal to the cleavage site and one pair on the inside of stem II are conserved (Ruffner *et al.*, 1990). The cleavage site is limited to 5′-NUX-3′ (where N and X denote any nucleotide and A, U, or C, respectively), although the sequence GUC is the preferable target sequence (Ruffner *et al.*, 1990; Shimayama *et al.*, 1995; Zoumadakis and Tabler, 1995).

Specificity of the reaction by a hammerhead ribozyme is crucial for cleavage at a specific site of target RNA. The cleavage specificity is determined by the hybridizing arms of the ribozyme, which anneal with the substrate in a complementary fashion to form stems I and III and lead to cleavage of the scissile phosphodiester bond. The length, base composition, and conformation of stems I and III as the substrate recognition helices could be essential factors for specific cleavage. So far, relatively little attention has been paid to the effects of base-pairing or specific nucleotides in stems I and III on the catalytic reaction compared to the function of stem II, which is involved in the actual catalysis. An absolute requirement for the two inner base pairs in stems I and III is known (Werner and Uhlenbeck, 1995) and the optimum length of stem I was suggested to be 5 or 6 bases (Hendry and McCall, 1996). However, there has been no systematic approach to illustrate the effects of base-pairing and length in stems I and III on the ribozyme reaction.

In this work, we tried to select hammerhead ribozymes with optimized structures of stems I and III, which were targeted to the human hepatitis B virus (HBV) X mRNA, by an *in vitro* selection method. The HBV is a small hepatotropic DNA virus causing acute and chronic

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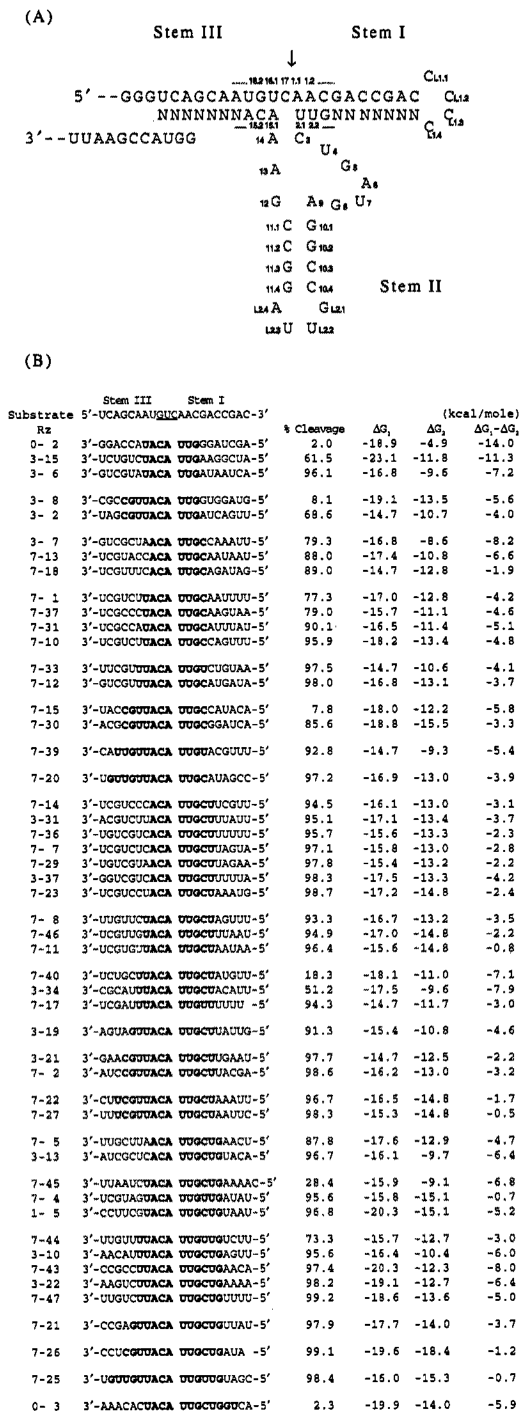

Rz	Sequence	% Cleavage	ΔG_1 (kcal/mole)	ΔG_2 (kcal/mole)	$\Delta G_1-\Delta G_2$ (kcal/mole)
0- 2	3'-GGACCAUACA UUGGGAUCGA-5'	2.0	-18.9	-4.9	-14.0
3-15	3'-UCUGUCUACA UUGAAGGCUA-5'	61.5	-23.1	-11.8	-11.3
3- 6	3'-GUCGUAUACA UUGAUAAUCA-5'	96.1	-16.8	-9.6	-7.2
3- 8	3'-CGCCGUUACA UUGGUGGAUG-5'	8.1	-19.1	-13.5	-5.6
3- 2	3'-UAGCGUUACA UUGAUCAGUU-5'	68.6	-14.7	-10.7	-4.0
3- 7	3'-GUCGCUAACA UUGCCAAAUU-5'	79.3	-16.8	-8.6	-8.2
7-13	3'-UCGUACCACA UUGCAAUAAU-5'	88.0	-17.4	-10.8	-6.6
7-18	3'-UCGUUUCACA UUGCAGAUAG-5'	89.0	-14.7	-12.8	-1.9
7- 1	3'-UCGUCUUACA UUGCAAUUUU-5'	77.3	-17.0	-12.8	-4.2
7-37	3'-UCGCCCUACA UUGCAAGUAA-5'	79.0	-15.7	-11.1	-4.6
7-31	3'-UCGCCAUACA UUGCAUUUAU-5'	90.1	-16.5	-11.4	-5.1
7-10	3'-UCGUCUUACA UUGCCAGUUU-5'	95.9	-18.2	-13.4	-4.8
7-33	3'-UUCGUUUACA UUGUCUGUAA-5'	97.5	-14.7	-10.6	-4.1
7-12	3'-GUCGUUUACA UUGCAUGAUA-5'	98.0	-16.8	-13.1	-3.7
7-15	3'-UACCGUUACA UUGCCAUACA-5'	7.8	-18.0	-12.2	-5.8
7-30	3'-ACGCGUUACA UUGCGGAUCA-5'	85.6	-18.8	-15.5	-3.3
7-39	3'-CAUUGUUACA UUGUACGUUU-5'	92.8	-14.7	-9.3	-5.4
7-20	3'-UGUUGUUACA UUGCAUAGCC-5'	97.2	-16.9	-13.0	-3.9
7-14	3'-UCGUCCCACA UUGCUUCGUU-5'	94.5	-16.1	-13.0	-3.1
3-31	3'-ACGUCUUACA UUGCUUUAUU-5'	95.1	-17.1	-13.4	-3.7
7-36	3'-UGUCGUCACA UUGCUUUUUU-5'	95.7	-15.6	-13.3	-2.3
7- 7	3'-UCGUCUCACA UUGCUUAGUA-5'	97.1	-15.8	-13.0	-2.8
7-29	3'-UGUCGUAACA UUGCUUAGAA-5'	97.8	-15.4	-13.2	-2.2
3-37	3'-GGUCGUCACA UUGCUUUUUA-5'	98.3	-17.5	-13.3	-4.2
7-23	3'-UCGUCCUACA UUGCUAAAUG-5'	98.7	-17.2	-14.8	-2.4
7- 8	3'-UUGUUCUACA UUGCUAGUUU-5'	93.3	-16.7	-13.2	-3.5
7-46	3'-UCGUUGUACA UUGCUUUAAU-5'	94.9	-17.0	-14.8	-2.2
7-11	3'-UCGUGUUACA UUGCUAAUAA-5'	96.4	-15.6	-14.8	-0.8
7-40	3'-UCUGCUUACA UUGCUAUGUU-5'	18.3	-18.1	-11.0	-7.1
3-34	3'-CGCAUUUACA UUGCUACAUU-5'	51.2	-17.5	-9.6	-7.9
7-17	3'-UCGAUUUACA UUGUUUUUU -5'	94.3	-14.7	-11.7	-3.0
3-19	3'-AGUAGUUACA UUGCUUAUUG-5'	91.3	-15.4	-10.8	-4.6
3-21	3'-GAACGUUACA UUGCUUGAAU-5'	97.7	-14.7	-12.5	-2.2
7- 2	3'-AUCCGUUACA UUGCUUACGA-5'	98.6	-16.2	-13.0	-3.2
7-22	3'-CUUCGUUACA UUGCUAAAUU-5'	96.7	-16.5	-14.8	-1.7
7-27	3'-UUUCGUUACA UUGCUAAUUC-5'	98.3	-15.3	-14.8	-0.5
7- 5	3'-UUGCUUAACA UUGCUGAACU-5'	87.8	-17.6	-12.9	-4.7
3-13	3'-AUCGCUCACA UUGCUGUACA-5'	96.7	-16.1	-9.7	-6.4
7-45	3'-UUAAUCUACA UUGCUGAAAAC-5'	28.4	-15.9	-9.1	-6.8
7- 4	3'-UCGUAGUACA UUGUUGAUAU-5'	95.6	-15.8	-15.1	-0.7
1- 5	3'-CCUUCGUACA UUGCUGUAAU-5'	96.8	-20.3	-15.1	-5.2
7-44	3'-UUGUUUUACA UUGUUGUCUU-5'	73.3	-15.7	-12.7	-3.0
3-10	3'-AACAUUUACA UUGCUGAGUU-5'	95.6	-16.4	-10.4	-6.0
7-43	3'-CCGCCUUACA UUGCUGAACA-5'	97.4	-20.3	-12.3	-8.0
3-22	3'-AAGUCUUACA UUGCUGAAAA-5'	98.2	-19.1	-12.7	-6.4
7-47	3'-UUGUCUUACA UUGCUGUUUU-5'	99.2	-18.6	-13.6	-5.0
7-21	3'-CCGAGUUACA UUGCUGUUAU-5'	97.9	-17.7	-14.0	-3.7
7-26	3'-CCUCGUUACA UUGCUGAUA -5'	99.1	-19.6	-18.4	-1.2
7-25	3'-UGUUGUUACA UUGUUGUAGC-5'	98.4	-16.0	-15.3	-0.7
0- 3	3'-AAACACUACA UUGCUGGUCA-5'	2.3	-19.9	-14.0	-5.9

Fig. 1. The *cis*-acting ribozyme pool, pool design, and selected ribozymes. (A) Secondary structure of the ribozyme pool. Random nucleotides are denoted by N. The cleavage site is marked by the vertical arrow. The number on each base is according to the numbering system of Hertel *et al.* (1992). (B) Selected ribozyme sequences, and their cleavage efficiencies and folding energies. Only the recognition sequences are shown. Bases involved in contiguous base-pairing are in boldface. Rz 0, Rz 1, Rz 3, and Rz 7 represent ribozymes cloned from the initial pool, and after the first, third, and seventh round selections, respectively. ΔG_1, and ΔG_2, folding energies without and with the constraints, respectively (see the text).

hepatitis, cirrhosis, and hepato-carcinoma in humans (Tiollais *et al.*, 1985). Among the 4 open reading frames designated S, C, P, and X in the HBV genome, the X has been known to encode a strong transcriptional *trans*-activator playing an essential role in hepatocellular carcinogenesis (Twu and Robinson, 1989). The suppression of HBV X gene expression from the infecting HBV could be a good strategy to inhibit the replication of the virus. A putative secondary structure of the X mRNA was proposed based on chemical and enzymatic determination of single- and double-stranded regions (Kim *et al.*, 1995). Since, in the secondary structure, the GUC sequence at positions 1679 to 1681 of the HBV genome is located in an extended single-stranded region, this sequence was chosen for a cleavage target site.

We applied *in vitro* selection (Gold *et al.*, 1995; Thomson *et al.*, 1996) as a powerful tool to isolate hammerhead ribozymes with optimized stems I and III, and obtained active *cis*-acting ribozymes having various structures in the stems.

Materials and Methods

Oligonucleotides The following oligonucleotides were used for DNA templates to produce a partially randomized hammerhead ribozyme pool or used as primers for polymerase chain reaction. Oligo 1, 5'-CCT**GGATCC**TTAATACGACTCACTATAGGGT-CAGCAATGTCAACGACCGACCCC-C-3' (the *BamHI* site for cloning and the T7 promoter sequence are in boldface and underlined, respectively); oligo 2, 5'-GC**GAATTC**CGGTACC-NNNNNNNTGTTTCGGCCTAACGGCCTCATCAGAACNNNNN-NNGGGGGTCGGTCGTT-3' (the *EcoRI* site for cloning is shown in boldface and the random nucleotides are represented by N); oligo 3, 5'-GCGAATTCCGGTACC-3'; oligo 4, 5'-CCTGGATCCTTAATACGA-3'.

Selection protocols The procedure for selection is diagrammed in Fig. 2. Oligos 1 and 2 were annealed by slowly cooling the mixture to 28°C after heating to 70°C. The annealed DNA (20 μg) was incubated with 10 units of T4 DNA polymerase in 100 μl of 10 mM Tris-HCl, pH 7.5, 0.5 mM dNTP, 1 mM ATP, and 2 mM DTT at 37°C for 3 h. The extended DNA product was purified by gel elution as previously described (Sambrook *et al.*, 1989) and used as the initial DNA templates for *in vitro* transcription to generate a partially randomized *cis*-acting ribozyme pool. *In vitro* transcription was carried out with 10 pmol of the DNA template and 45 units of T7 RNA polymerase in 100 μl of 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.5 mM NTP, and 10 mM DTT. The transcription mixture was incubated at 37°C for 3 h. The reaction was terminated by adding EDTA to a final concentration of 15 mM. The transcripts were electrophoresed in an 8% polyacrylamide–7 M urea gel. Cleaved RNA migrating at about 75 nucleotides was eluted from the gel. Oligo 3 was designed to anneal with the cleavage product and produce the complementary DNA by primer extension with reverse transcriptase. Oligo 3 (50 pmole) and the cleaved RNA were heated at 90°C for 2 min and cooled slowly to 42°C. Primer extension was carried out with

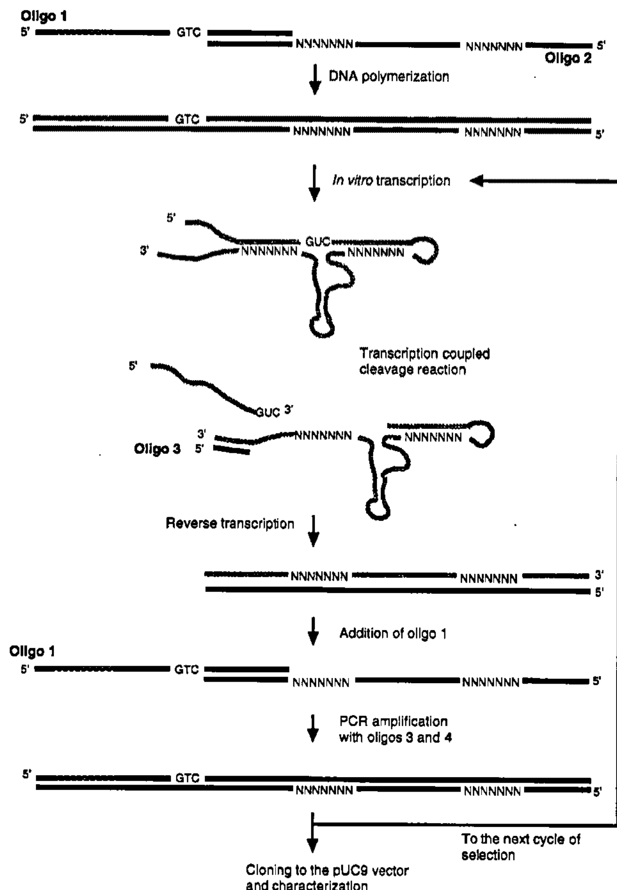

Fig. 2. Scheme of *in vitro* selection. A partially randomized RNA pool was produced from the DNA templates. The cleaved RNAs were reverse-transcribed and the resulting cDNAs were amplified by the polymerase chain reaction (PCR). The double-stranded DNAs were utilized as templates in subsequent selection cycles or were cloned. The solid bar and dotted bar indicate DNA and RNA, respectively. The slashed bar denotes the T7 promoter.

the annealed product and 5 units of AMV reverse transcriptase in 30 μl of 34 mM Tris-HCl, pH 8.3, 50 mM $MgCl_2$, 0.5 mM dNTP, and 5 mM DTT at 42°C for 2 h. The mixture was then heated to 75°C for 10 min to inactivate the reverse transcriptase and cooled on ice for the polymerase chain reaction. The full-length double-stranded DNA was subsequently produced by the polymerase chain reaction with 50 pmole each of oligos 3 and 4, 4 pmole of oligo 1, and 5 units of *Taq* DNA polymerase in 100 μl of 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1.0% Triton X-100, 0.2 mM dNTP. Oligo 1 restored the sequence including the *BamHI* site and the T7 promoter sequence which was removed by the cleavage.

The amplified DNA was purified by gel electrophoresis. The DNA was used as the template for *in vitro* transcription to generate an RNA pool for the next selection. Multiple rounds of successive selection were carried out to enrich ribozymes with high cleavage efficiency except for the following changes of the conditions: starting from the fourth round selection, the concentration of template DNA was diluted to one tenth in the *in vitro* transcription reaction to reduce the possibility of *trans* cleavage; the transcription reaction time was reduced from 3 h (for the first to third round selections) to 1 h (for the fourth round), 50 min (the fifth round), 40 min (the sixth round), or 30 min (the seventh round).

After the selection, the amplified DNA was digested with *BamHI* and *EcoRI* and cloned into the pUC9 plasmid. Individual ribozyme sequences were determined by DNA sequencing analysis with a pUC9 reverse sequence primer using the TaqTrack sequencing kit (Promega).

Cleavage efficiency of ribozymes The cleavage efficiency of *cis*-acting hammerhead ribozymes was determined in a transcription-cleavage coupled reaction. DNA templates for *in vitro* transcription were the DNA amplified by the polymerase chain reaction after the selection, or plasmid DNAs linearized with *EcoRI*. *In vitro* transcription was carried out with 0.1 pmol of DNA using 7.5 units of T7 RNA polymerase in a transcription reaction at 37°C for 30 min in 10 μl of 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.5 mM each of ATP, GTP, and UTP, 20 μM [α-^{32}P]CTP, 10 mM DTT, and 1 unit of RNasin RNase inhibitor. The reaction was terminated by adding EDTA (final concentration, 15 mM). The reaction mixture was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and RNA was ethanol-precipitated and analyzed in an 8% polyacrylamide gel containing 8 M urea. Relative amounts of the full-length transcript and the cleaved transcript were estimated with a Molecular Dynamics PhosphorImager. The experiment was carried out in duplicate and the cleavage efficiency was determined according to the following formula: % cleavage = 100 P/(P + S), where S and P stand for the relative amounts of intact transcripts and cleaved RNAs, respectively.

Results and Discussion

A pool of *cis*-acting hammerhead ribozymes having various structures of stems I and III was constructed (Fig. 1A). The hammerhead ribozyme pool was composed of the target RNA containing the HBV sequence from nucleotides 1669 to 1691 (Kim *et al.*,1995), the consensus core sequences, and each substrate recognition arm composed of the proximal 3 bases complementary to the target and the distal 7 randomized bases. The proximal 3 bases are known to be essential for the cleavage reaction to occur (Werner and Uhlenbeck, 1995; Zoumadakis *et al.*, 1994), but the effect of the distal base pairings has not been well studied. The substrate region was connected to the ribozyme by a tetraloop 5′-CCCC-3′ sequence. Since the tetraloop sequence does not make the stable tetraloop structure (Woese *et al.*,1990), the possibility of stabilization of stem I by a stable loop would be low in this construct.

In brief, the protocol of selection included *in vitro* transcription of a RNA pool from the partially randomized DNA templates and isolation on an 8% denaturing polyacrylamide gel of shorter cleavage products formed in an *in vitro* transcription-coupled cleavage reaction (Fig. 2). The cleaved RNA was reverse-transcribed using oligo 3. The resulting cDNA was amplified by the polymerase

chain reaction using oligos 3 and 1 (which restores the sequence lost by the cleavage including the T7 RNA polymerase promoter), and then using oligos 3 and 4. The double-stranded DNA was utilized as a template in subsequent selection cycles or for cloning.

Cleavage efficiencies of the selected RNA pools were determined (Fig. 3). RNAs after the first to seventh round of selection were cleaved with 0.41, 2.7, 18.4, 26.5, 56.0, 74.0, and 74.5% efficiencies, respectively, suggesting that the selected RNA pool was saturated with well-cleavable RNAs after six cycles of selection.

Fifty ribozymes were cloned among the cDNAs from the random, first, third, and seventh RNA pools and then sequenced. *In vitro* transcription from each cloned DNA was carried out and the cleavage efficiency of the transcripts was determined (Fig. 1B). Since the cleavage efficiency was determined in the transcription-coupled cleavage reaction, all ribozymes having the cleavage rate higher than the transcription rate would show about the same and nearly complete cleavage. Therefore, cleavage efficiencies between those ribozymes could not be compared by this experiment. The value of ΔG for the most stable secondary structure of each ribozyme was calculated by the MFOLD program, which was developed by Zuker (Jaeger *et al.*, 1989). Given no constraints, most of the secondary structures did not show the expected hammerhead structure. When constraints were added which forced formation of base-pairs $U^{16.1}\cdot A^{15.1}$, $G^{16.2}\cdot C^{15.2}$, $U^{16.3}\cdot A^{15.3}$, $A^{1.1}\cdot U^{2.1}$, $A^{1.2}\cdot U^{2.2}$, and $C^{1.3}\cdot G^{2.3}$, and which prohibited C^{17} from forming a base-pair, the resulting structures maintained the hammerhead structure. The secondary structures predicted with and without the constraints differed in their energy values. Most active ribozymes had energy values between −20 and −15 kcal/mol when calculated without the constraints, while the energy values calculated with constraints were between −15 and −10 kcal/mol, suggesting that the most stable structure of a given ribozyme is not an active one. The difference between the two energy values was plotted against cleavage efficiency (Fig. 4). The ribozymes with the smaller energy difference showed a tendency to have the better cleavage efficiency, implying that the conformation leading to the cleavage reaction prevails in those ribozymes, possibly due to the small energy gap between the active conformation and the stable but inactive conformations. It should be noted, however, that the calculated energies may differ from the actual energies, particularly because of the presence of the unique structure in the catalytic core of the hammerhead ribozyme (Pley *et al.*, 1994). The effect of alternative conformations of virusoid RNA on self-cleaving activity has been reported (Forster and Symons, 1987). Plus and minus strands of vLTSV only self-cleave after snap-cooling and remain inactive after slow-cooling. Presumably, slow cooling allows the formation of the native, inactive conformation, while snap-cooling induces formation of an alternative, active conformation.

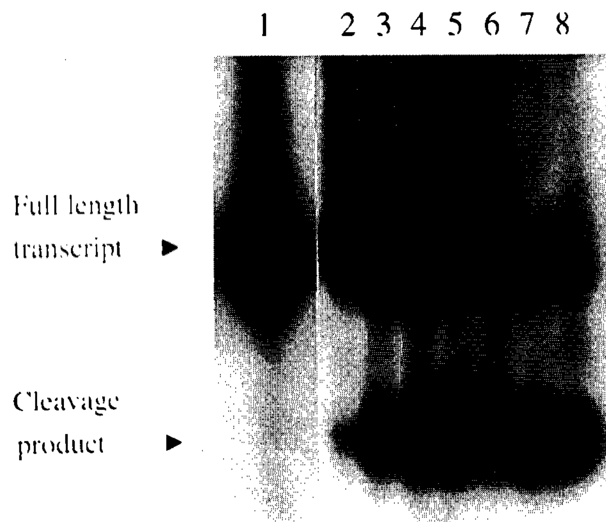

Fig. 3. Cleavage efficiencies of selected pools. The cleavage efficiencies of selected pools were determined in a transcription coupled cleavage reaction in the presence of [α-^{32}P] CTP. The transcripts were electrophoresed on an 8% polyacrylamide gel containing 8 M urea. (1: the initial pool, 2: the first pool, 3: the second pool, 4: the third pool, 5: the fourth pool, 6: the fifth pool, 7: the sixth pool, 8: the seventh pool)

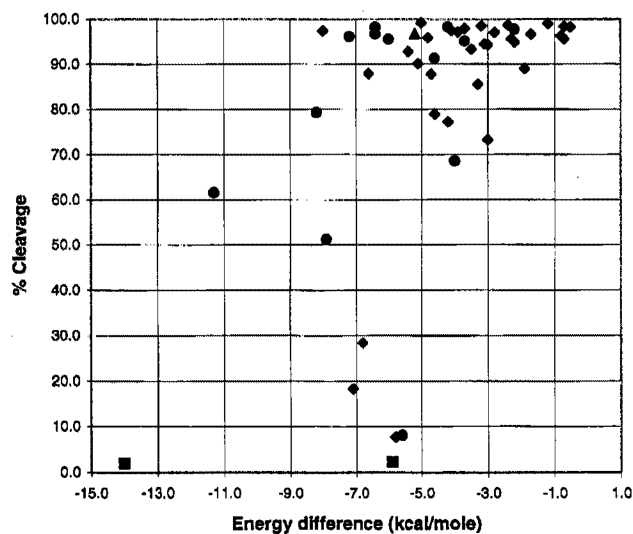

Fig. 4. Effects of the difference between folding energies with and without the constraints on cleavage. ■: the initial pool, ▲: the first pool, ●: the third pool, ◆: the seventh pool.

The cloned ribozymes were grouped with respect to the length of stems I and III (Fig. 1B), which was defined as the number of contiguous base pairs just adjacent to the scissile bond. All ribozymes with more than 90% cleavage efficiency have a 4, 5, or 6 base-paired stem I. Considering

the probability of selection in the random pool, ribozymes with stem I having 4 bases were expected to be more common (P_{exp} = 0.4), but a large population of the ribozymes turned out to have 5 or 6 bases in stem I (P_{exp} = 0.09 for 5 bases; 0.02 for 6 bases). On the other hand, the length of stem III varied between 3 to 9 bases. Although the population of ribozymes with shorter stem III was larger than that of longer stem III, this does not mean that ribozymes with shorter stem III had a better cleavage activity because the selection probability of ribozymes with shorter stems in the random pool should be higher than those with longer stems (P_{exp} = 0.8 for 3 bases; 0.2 for 4 bases; 0.05 for 5 bases; 0.008 for 6 bases; 0.006 for 7 bases; 0.001 for 8 bases; 0.0001 for 9 bases). Therefore, the optimum length of stem I is 5 or 6 bases, while that of stem III appears to be rather flexible. Many ribozymes with stem III of 7 to 9 bases were selected despite the much lower probabilities, although any ribozymes with a stem I of more than 6 bases were not found. This suggests that stem III requires relatively long stems for efficient cleavage compared to stem I. These data can account for previous studies showing that stem I needs to be relatively short or weak and stem III can be much longer and/or more stable for rapid cleavage of a substrate by hammerhead ribozymes. In the context of a very long stem III, stem I can be reduced to as few as 3 bp without loss of activity (Zoumadakis *et al.*, 1994). In the context of relatively short substrate binding stems (Werner and Uhlenbeck, 1995), mismatches close to the core in stem I are tolerated much more than in stem III, where mismatches in any of the four innermost base pairs cause a significant decrease in the reaction rate. Recently, Hendry and McCall (1996) have shown that the substrate in the ribozyme-substrate complex with helix I of 5 bases and helix III of 10 bases is cleaved more rapidly than those forming with helix I of 10 bases and helix III of 5 bases.

There are several ribozymes which have the same length of stems I and III but show a large difference in cleavage efficiency (Fig. 1B). In most cases, the likely cause for the difference can be attributed to the energy difference between the active and inactive conformations (as discussed above) because the energy difference is inversely correlated with cleavage efficiency. Since some ribozymes such as Rz7-40 (5 bases in both stems) and Rz7-44 (6 and 5 bases in stems I and III, respectively) deviate from this simple rule, their cleavage efficiencies cannot be explained by the energy difference. These ribozymes could have sequence contexts or other structural elements which may affect a working tertiary structure for the cleavage reaction.

The specificity of the hammerhead ribozyme reaction is crucial for cleavage of a specific mRNA in a large pool of intracellular RNA. The simplest model is that the specificity of cleavage depends on the difference in the affinities of binding between the correct substrate and RNA sequences differing from the target sequence. Affinity is believed to be determined by the length and base composition of stems I and III as the substrate recognition helices. In our *in vitro* selection experiment, however, a variety of *cis*-acting ribozymes having various base-pairing, even 3 and 4 base pairs or vice versa, of stems I and III were selected. This implies that a given ribozyme to a specific target can cleave nonspecific targets which are recognized by a part of the hybridizing arms of the ribozyme, and that the specificity can not be secured only by increasing the length of the recognition helices. Therefore, other means of affecting the specificity, for example by using an antisense RNA to a different site in the same target RNA, should be considered for ribozyme design.

Our eventual goal is to develop *trans*-acting hammerhead ribozymes which can be used as therapeutic agents against HBV infection. Information about the effects of base-pairing and structure of the substrate recognition helices on the self-cleavage reaction of hammerhead ribozymes can be used to refine the design of the *trans*-acting ribozymes.

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