In Vitro Selection of Hairpin Ribozymes Activated with Short Oligonucleotides

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ABSTRACT: We have carried out an *in vitro* selection to obtain an allosteric hairpin ribozyme, which has cleavage activity in the presence of an exogenous short oligonucleotide as a regulator. Random sequences were inserted in a region corresponding to the hairpin loop of the ribozyme. After 12 rounds of selection, DNA templates were cloned. Of a total of 34 clones, 18 contained the same sequence, and the obtained hairpin ribozymes showed the cleavage activity specifically in the presence of the regulator oligonucleotide. All of the clones contained sequences complementary to the regulator oligonucleotide. The ribozymes with high cleavage activities gained characteristic hairpin loops at the random domain, which were similar to each other. In the absence of the oligonucleotide, the loop domain within the allosteric ribozyme probably forms a slipped hairpin loop, and the complementary sequence, with the regulator oligonucleotide located at the single stranded loop, would allow easy access of the oligonucleotide. The binding of the regulator oligonucleotide triggers a structural change of the hairpin loop to form an active conformation. Furthermore, we constructed an allosteric hammerhead ribozyme by introducing the characteristic hairpin loop. The modified hammerhead ribozyme was also changed to an allosteric ribozyme, which was activated by the addition of the regulator oligonucleotide. The characteristic hairpin loop, which was proved to be regulated by an exogenous oligonucleotide in this report, may be used to control RNA functions in various fields.

Both hammerhead and hairpin ribozymes, which are well-known as small catalytic RNAs, can catalyze either cleavage or ligation of RNA with sequence specificity. Recently, those ribozymes have been applied to gene therapy, by cutting the mRNAs of target genes (*1*, *2*), and to gene discovery (*3*). These ribozymes, which compose the catalytic core of viral satellite RNA, normally do not have the ability to regulate their catalytic activities. Therefore, allosteric control has been applied to the ribozymes (*4*, *5*).

The hammerhead ribozyme consists of three helices (stems I, II, and III) and an internal loop region at a junction of the three helices. Most of the bases essential for the catalysis are located in the internal loop region. The three-dimensional structure of the hammerhead ribozyme has been revealed by X-ray crystallography (*6*, *7*). The sequence of stem II is variable, but the G10.1/C11.1 base pair adjacent to the catalytic core is important for the cleavage activity. Stem II can also be replaced by non-nucleotidic linkers (*8*). Breaker and co-workers carried out an *in vitro* selection of stem II and constructed a series of allosteric ribozymes responding to small molecules, such as ATP (*9*) or cyclic nucleotides (*10*). Furthermore, they succeeded in constructing an array addressing their allosteric ribozymes (*11*). Taira and co-workers also reported four pieces of ribozyme that could be activated with an oligoribonucleotide (*12*). The catalytic site of the ribozyme consisted of two-stranded RNAs. Recently, we have substituted stem II with a non-self-complementary loop sequence, and the cleavage activity was specifically induced by the addition of a short oligoribonucleotide complementary to the single stranded loop (*13*). Since a pseudo-stem II was formed between the exogenous oligonucleotide and the ribozyme, the ribozyme was activated. The activation by the oligoribonucleotide was performed with sequence specificity.

The hairpin ribozyme has two stem−loop domains, and both domains involve two helices and one internal loop (helices 1, 2, and loop A in domain I; helices 3, 4, and loop B in domain II) (*14*−*16*). Essential bases for the cleavage activity are located in the two internal loops. It has been proven that the bent structure is an active conformation (*17*, *18*). Furthermore, the domain docking of the hairpin ribozyme was investigated in detail, using fluorescence measurements and gel mobility shift assays (*19*−*21*). Conformational changes in the internal loops are thought to occur during the docking process (*22*, *23*). Although helix 4 is essential for the cleavage activity, the bases included in the region are variable. The stabilization of helix 4 increased the cleavage activity of the hairpin ribozyme (*24*). The formation of helix 4 is thought to stabilize the noncanonical base pairs in loop B. An NMR analysis of each domain has been reported (*25*, *26*). Recently, an X-ray analysis of the hairpin ribozyme has been reported (*27*). The three-dimensional structure revealed the sharp bent structure of the ribozyme and an interesting interaction between the two domains. The global conformation results were consistent with previous investigations.

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The hairpin ribozyme has not been modified into an allosteric enzyme responding to small molecules, in contrast to the hammerhead ribozyme. We now report the construction of an allosteric hairpin ribozyme, which is activated by a short oligonucleotide. The allosteric hairpin ribozyme contains a characteristic hairpin loop, with a structure that changes upon binding with the regulator oligonucleotide. Since the transmitter module for the signal passing showed the possibility of the allosteric modification of another ribozyme, the hairpin structure may be applied to the allosteric control of other functional RNA molecules.

MATERIAL AND METHODS

Preparation of DNA. All primers were synthesized with a DNA·RNA synthesizer using the phosphoramidite method and were purified by HPLC using a reverse phase column. An initial pool template (LRP1) including random 20-base sequences was also synthesized as the primers, and was then purified by fractionation on an 8% polyacrylamide gel (acrylamide/bisacrylamide, 19:1) containing 8 M urea. The double-stranded template of the initial pool was prepared from LRP1 and UP1. LRP1 (200 pmol) and UP1 (240 pmol) were heated at 90 °C for 5 min and were then cooled to room temperature to anneal both strands. Then, an elongation reaction was carried out to prepare complete double strands. The reaction mixture (100 μL) contained T7 sequenase DNA polymerase (6.5 units), 5 mM DTT, 0.37 mM NTP, 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and 50 mM NaCl. After the reaction, the solution was incubated at 37 °C for 2 h, and extracted with phenol and phenol−chloroform, and then precipitated with ethanol. The double strands were isolated from a nondenaturing 10% polyacrylamide gel (acrylamide/bisacrylamide, 29:1). After phenol, phenol−chloroform, and ethanol extractions, the double strands of the initial pool were dissolved in TE buffer (30 mL, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA·Na_2). The sequences of UP1 and LRP1 are 5′d(CGGCGAATTCTAATACGACTCACTATAGGGAAACAGAGAAGTCAACCAGAGAA)3′, and 5′d(GAGCTGGATCCAAACAGGACTGTCAGGGGGGTACCAGGTAATATAC (N_{20}) TGTTTCTCTGGTTGACTTCTCTG) 3′, respectively.

Transcription. RNA was transcribed from the template DNA by using an AmpliScribeTM transcription kit. The reaction mixture contained 7.5 mM NTP, 10 mM DTT, T7 RNA polymerase (AmpliScribeTM; 2 μL), [α-^{32}P]UTP, and template DNA (2 pmol for the initial round). After an incubation at 37 °C for 2 h, the solution was treated with DNase I (1 MBU; AmpliScribeTM) at 37 °C for 15 min. Then, the solution was desalted with NAP 5 (Pharmacia) and precipitated with ethanol. The RNA was dissolved in distilled water.

Selection. The transcribed RNA (20 pmol) was dissolved in the cleavage buffer (40 mM Tris-HCl (pH7.5), 12 mM $MgCl_2$, 2 mM spermidine-3HCl: 20 μL), heated at 65 °C for 2 min, and then immediately transferred to an ice bath. The solution was incubated at 37 °C, and then loading solution (50 mM EDTA·2Na, 10 M urea) was added to stop the reaction. The concentration of RNA was 1 μM in G0 to G6 (G0−G6), 2.5 μM in G7−G10, and 4 μM in G11. The reaction time for negative selection was 20 h for G0−G6, 30 min for G7−G9, and 10 min for G10−G11. The negative selections in G7−G9 were repeated twice. After the negative selections, denaturing 10% polyacrylamide gel electrophoresis (PAGE) was carried out, and the uncleaved products were purified from the gel. The RNA recovered from the gel slice was precipitated with ethanol, and then was dissolved in distilled water. The uncleaved RNAs were used for positive selection. RNA molecules that were recovered from the first negative selection (negative selection 1) in G7−G9, were again dissolved in the cleavage buffer [40 mM Tris-HCl (pH7.5), 12 mM $MgCl_2$, 2 mM spermidine-3HCl: 20 μL], and the negative selection was repeated (negative selection 2). The reaction conditions and the purification procedures were the same as those for first negative selection. The uncleaved RNAs were used for positive selection.

The inactive RNAs in the negative selection, which were dissolved in the cleavage buffer (20 μL) containing m7G (50 μM), were heated at 65 °C for 2 min, and then were immediately transferred to an ice bath. The reaction solution was incubated at 37 °C for the positive selection. The reaction times of the positive selections were gradually decreased, from 20 h for G0 to 10 min for G11. After the loading solution was added to the aliquots to stop the cleavage reactions, denaturing 10% polyacrylamide gel electrophoresis was carried out to separate the cleaved products from the inactive molecules. The cleavage products were recovered from the gel, precipitated with ethanol, and then dissolved in distilled water.

The purified RNA was used for reverse transcription in a reaction containing LP2 primer (20 μM), M-MLV-reverse transcriptase (10 units/μL), 10 mM DTT, 0.5 mM dNTPs, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. After the reverse transcription was carried out at 37 °C for 1 h, the reaction solution was heated at 94 °C for 5 min to inactivate the reverse transcriptase. To the reaction mixture (10 μL) containing the cDNA, AmpliTaq DNA polymerase buffer (×10; 0.5 μL), 2.5 mM dNTPs (1.6 μL), UP2 (20 pmol), LP2 (20 pmol) primers, and AmpliTaq DNA polymerase (5 units, 1 μL) were added, and then PCR was carried out. The thermal cycle program consisted of 94 °C for 60 s, 55 °C for and 75 s, 72 °C for 120 s, and the cycle was repeated 30 times. After extractions with phenol, phenol−chloroform, and chloroform, and precipitation with ethanol, the precipitate was dissolved in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA-Na_2). The DNA templates were used for the following selection. The sequences of UP2 and LP2 were 5′CGGCGAATTCTAATACGACTCACTCACTATAGGGAAACAG3′ and 5′GAGCTGGATCCAAACAGGACTGTCAGGGGGGTA3′, respectively.

Cleavage Reaction of G12. The G12 RNA was not subjected to the selection procedures, and the cleavage activity was investigated in either the presence or absence of m7G. G12 RNAs (1 μM) were incubated at 37 °C in the presence of m7G (50 μM) after heat denaturing at 65 °C for 2 min. Aliquots were taken from the reaction solution at time intervals and were added to the stop solution (50 mM EDTA-Na_2, 10 M urea).

Cleavage Reaction of RNA Molecules. RNA populations displaying an oligonucleotide-dependent self-cleavage were cloned (TOPO TA cloning kit, Invitrogen), and sequenced (Big Dye Terminator Ready Reaction Mix, PE Applied Biosystems). Double-stranded DNA templates were prepared from plasmid DNA by PCR amplification using primers UP2

and LP2. The double-stranded DNA templates purified from the gel were dissolved in TE solution (10 mM Tris-HCl pH8.0, 1 mM EDTA-Na_2) after desalting. RNAs were transcribed from the DNA templates as described above. Each RNA molecule (16 pmol) and m7G (200 pmol), which were separately dissolved in 8 and 10 μL of reaction buffer, respectively, were heated at 90 °C for 2 min and then immediately transferred to an ice bath. M7G (8 μL) was added to the ribozyme RNA to start the reaction, which was incubated at 37 °C. Aliquots were taken from the reaction solution and were added to the loading solution to stop the reactions. Electrophoresis on a 10% polyacrylamide gel containing 8 M urea electrophoresis was carried out, and then the radioisotope activity was measured by an imaging analyzer (BAS2000). Observed rate constants were calculated using the equation $S/(L + S) = a(1 - \exp(-k_{obs}t))$, in which S is the concentration of cleaved products, L is the concentration of the full-length transcripts, t is time, and a is the percentages of active molecules versus total RNA molecules.

The dissociation constant and the cleavage rate constants of the c1/m7G complex were measured from the observed rate constant (k_{obs}) values for various m7G concentrations. The observed rate constants of the c1/m7G complex were calculated as described above. The concentration of c1 was 1 μM, and that of m7G was varied from 0 to 100 μM. The dissociation constant (K_d) and the highest chemical step ($k_{RLO(+)}$)[1] with a saturating amount of the regulator oligonucleotide were determined from the equation $k_{obs} = (k_{RLO(-)}K_d + (k_{RLO(+)}[m7G])/(K_d + [m7G])$, as reported previously (*13*, *28*). The $k_{RLO(-)}$ value was determined by the cleavage reaction of c1 in the absence of m7G ($k_{RLO(-)} = 0.0033$ min^{-1}). $k_{RLO(+)}$ and $k_{RLO(-)}$ are the cleavage rate constants with a saturating amount of m7G and without m7G, respectively.

Cis-Cleavage Reaction during Transcription from the DNA Template. The DNA template (2.5 nM), 30 μM regulator oligonucleotides, 0.5 mM each NTP, 40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine-trihydrochloride, 5 mM DTT, 0.5 μCu/μL [α-^{32}P]-UTP, and T7 RNA polymerase (2 μL) from the AmpliScribe T7 kit were incubated in a total volume of 20 μL at 37 °C. Aliquots were taken at time intervals and were added to loading solution (10 M urea, 50 mM EDTA-Na_2) to stop the cleavage reaction. The products were analyzed on a 10% polyacrylamide gel containing 8 M urea. The cleavage rate constants were determined by fitting the data by a least-squares method to the following equation as described previously (*29*, *30*), $L/(L + S) = (1 - b)((1 - \exp(-k_{cis}t))/k_{cis}t) + b$, where L is the concentration of the full-length transcripts, S is the concentration of cleaved transcript, t is time, b is the percentages of the inactive molecule, and k_{cis} is the rate constant for cis-cleavage.

Trans-Cleavage Reaction after Cis-Cleavage during Transcription. The DNA template for c1 (2.5 nM), 0.5 mM each NTP, 40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine-trihydrochloride, 5 mM DTT, 0.5 μCu/μL [α-^{32}P]-UTP, and T7 RNA polymerase (2 μL) from the AmpliScribe T7 kit were incubated in the absence or presence of 0.05 or 0.5 μM RLO at 37 °C. After 1 h of transcription, the reaction solution (total volume of 20 μL) was heated at 95 °C for 2 min to terminate the transcription. To the reaction solution, 5′-end labeled S1 (4 μM; 5 μL), which was dissolved in cleavage buffer (200 mM Tris-HCl (pH 7.5), 60∼2000 mM $MgCl_2$, 10 mM spermidine-trihydrochloride) was added, and the reaction solution was incubated at 37 °C to start the trans-cleavage reaction. The reaction solution contained 0.8 μM S1 and 12, 30, or 100 mM $MgCl_2$. Aliquots were taken at time intervals and were added to loading solution (10 M urea, 50 mM EDTA-Na2) to stop the cleavage reaction. The cleavage products were analyzed on a 20% polyacrylamide gel containing 8 M urea.

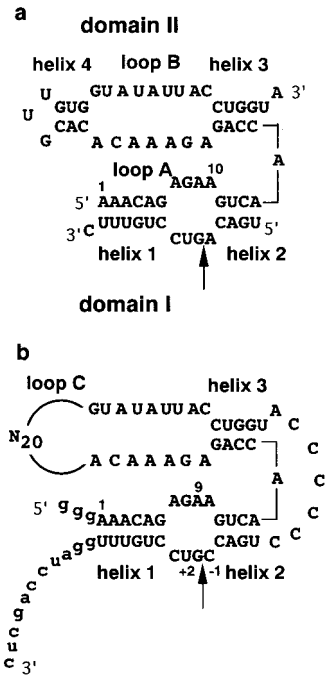


FIGURE 1: Secondary structures of the wild-type hairpin ribozyme (a, trans-cleavage system) and the ribozyme with a 20-nucleotide random-sequence domain (N_{20}) (b, cis-cleavage system). Arrows and lower characters indicate cleavage sites and sequences derived from the DNA template, respectively.

Cleavage Reaction with Modified Hammerhead Ribozyme. The 5′-end labeled substrate RNA (S1; 5 nM) was incubated with HH44PH (0.1 μM) in the presence of regulator oligonucleotides (m7G or r7G; 2 μM) at 37 °C. The reaction buffer contained 50 mM Tris-HCl (pH7.5), 50 mM NaCl, and 25 mM $MgCl_2$. Aliquots were taken from the reaction solution at time intervals and then were added to the stop solution (50 mM EDTA-Na_2, 10 M urea). Electrophoresis on a denaturing 20% polyacrylamide gel containing 8 M urea was carried out, and then the radioisotope activity was measured by an imaging analyzer (BAS2000).

RESULTS AND DISCUSSION

In Vitro Selection. Although both the hairpin loop and helix 4 of the hairpin ribozyme (Figure 1a) are absolutely required for the cleavage activity, the sequence of the helix−loop region is variable. Therefore, we planned to introduce a 20-nucleotide random sequence into the helix−loop (Figure 1b), and prepared DNA templates, which were expected to express 10^{12} species of hairpin ribozyme molecules. The random sequence region is referred as the loop C.

[1] Abbreviations: RLO, regulator oligonucleotide.

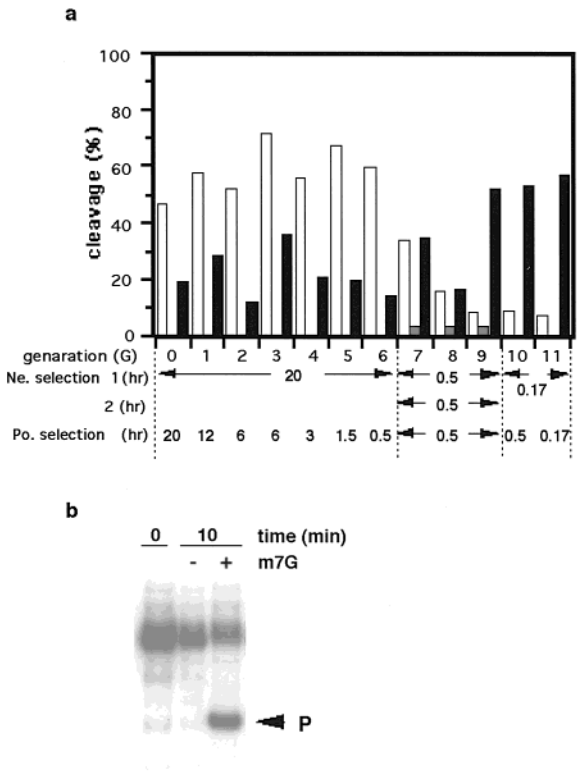


FIGURE 2: Progress of in vitro selection. (a) Percentages of self-cleavage activities for each round of selection. Reaction times (h) for negative (Ne.) and positive (Po.) selections are indicated below the horizontal axis. Self-cleavage activities of negative 1, 2, and positive selections are shown in white, gray, and black bars, respectively. (b) Analysis of the cleavage reaction of G12. G12 RNA transcripts were incubated without (−) or with (+) m7G for 10 min. P indicates the 5′-side cleavage product.

After transcription from the DNA library, we carried out negative selection by incubating the transcripts in the absence of a regulator oligonucleotide (RLO). As the RLO, a 2′-*O*-methyl oligoribonucleotide (2′-OMe(GAGUGAG)rG) consisting of eight nucleotides, in which only the 3′-end was a guanosine, was used. Previously, we reported that the regulator oligonucleotide (m7G) had activated a modified hammerhead ribozyme, which had not been developed by *in vitro* selection. After the cleavage reaction without m7G, uncleaved transcripts were isolated by denaturing polyacrylamide gel electrophoresis (PAGE). Those RNA molecules were then incubated in the presence of m7G (positive selection), and the 5′-side fragments from the cleavage products were purified by PAGE. The active molecules in the positive selection were converted to DNA by reverse-transcriptase, followed by PCR amplification. The double-stranded DNAs were used as the templates for the following transcription. The set of both negative and positive selections was repeated. The cleavage percent and the reaction time for each round are shown in Figure 2a.

Until G6, the time for the negative selections was 20 h, while that of the positive selections was gradually shortened from 20 h to 30 min. However, the self-cleavage percent in the negative selections did not decrease for first seven generations (G0−G6). On the other hand, the self-cleavage percent in the positive selections gradually increased, but no significant increase was observed. As the reason for this result, we thought that the ribozyme molecules, which were independently active in the presence of the RLO, had not been removed completely. Therefore, we carried out a secondary negative selection (negative selection 2) from G7 to remove as many active molecules as possible. The reaction times of the negative 1, 2, and positive selections were each 30 min. As a result of this negative selection, the active molecules in the negative selections were decreased efficiently by the repeated negative selections. The percent of the cleavage for the negative selection 1 of G9 was about 8% for 30 min, while that for the positive selection was 52% in the presence of the RLO. Since we thought that most of the active molecules without the regulator oligonucleotide were removed in G9, the negative selection 2 was omitted in the following selections. To achieve a more stringent response to the regulator oligonucleotide, both the negative and positive selection times were decreased to 10 min from G10. The self-cleavage percent of the negative and positive selections for G12 were 3.2 and 75%, respectively, during 10 min reactions (Figure 2b).

Table 1: Sequence and Self-Cleavage Rate Constant of Each Clone

clone	sequence[a]	number of clones	k_{obs} (−)[b]	k_{obs} (+)[b]	k_{obs} (+)/k_{obs} (−)
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20				
c1	5′ AUCUGCAUCUCACUCGAGAU 3′	18	0.0033	0.69	207
c2	UCA-CCCGCUCACUCAGUGG	1	0.0019	0.66	345
c3	AUC-GCAUCUCACUCGAGAU	1	0.0023	0.74	327
c4	GUGUACGCCUCAUUCUGCGC	1	0.0022	0.56	257
c5	UCUAAUACUCACUCUGCUGA	1	0.0026	0.55	208
c6	UUACAUACUCACUCAUGUGA	1	0.0038	0.55	143
c7	ACUUGC-ACUCACUCUGAGU	1	0.0024	0.54	223
c8	UCUUAUACUCACUCUAUUGA	1	0.0028	0.53	190
c9	UUUCGCUUCCUCACCGGGAA	1	0.0026	0.34	133
c10	UGCCUUUGGAUCCCACUCCA	1	0.035	0.28	7.9
c11	CCAUGUGCUCACUGCGUGUG	1	0.0031	0.25	80
c12	UCCAUGCUCACUCAAAGUGA	1	0.0021	0.25	120
c13	UCUAUGUACUCACCCAGUGA	1	0.0039	0.20	51
c14	ACUUUACGUCACUCUAAGGC	1	0.0022	0.12	53
c15	CUAUGCCUCCUCAUCCAGAG	1	0.0018	0.15	87
c16	UGCUGUAUCCUCACGGAGCA	1	0.0030	0.14	46
c17	CUAUAUCUACCUCGCCGUAG	1	0.0018	0.054	30
total		34			

[a] The 20-nucleotide randomized sequences are numbered from 1 to 20. Sequences complementary to m7G are indicated by red characters. [b] Rate constants were determined from the cleavage reactions containing the ribozyme (c1∼c17; 1 μM) and m7G (10 μM). k_{obs} (+) and k_{obs} (−) indicate the observed self-cleavage rate constant of each clone in either the presence (+) or absence (−) of m7G.

Sequences of Clones. We cloned G12, and investigated the sequences of 34 colonies. Loop C, corresponding to the 20-nucleotide random sequence in the ribozyme pool, is numbered from the 5′ to 3′ direction, and the sequences of the clones are listed in Table 1. Interestingly, clone 1 (c1) was most frequently detected (18 clones), and the others were found to be unique clones. One base deletion within loop C was observed in c2, c3, and c7. The c3 clone seems to be generated from the deletion of uridine 4 (U4) from c1, because the other sequences, except for U4, are the same as c1. All of the clones included the sequence complementary to the regulator oligonucleotide (m7G), and most of these complementary sequences were located from the center to the 3′-side of the loop C (red characters in Table 1).

In the wild-type hairpin ribozyme, helix 4, which is essential for the cleavage activity, stabilizes the noncanonical base pairs in loop B, such as A(+26)·G(+36) and C(+25)·U(+37). No mutations were detected within loops A and B of all clones. Thus, the active structure of loop B is likely to be the same as that of the wild-type ribozyme. In other words,

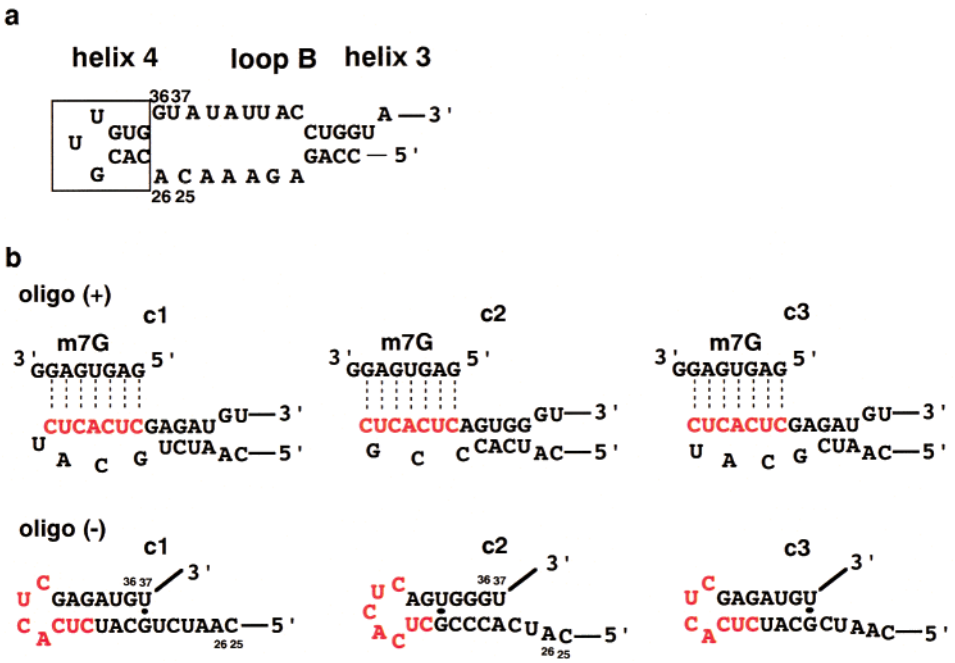


FIGURE 3: Secondary structures of the hairpin loops of allosteric ribozymes. (a) Domain II of the wild-type hairpin ribozyme. The hairpin loop of the wild-type ribozyme is surrounded by a square. (b) Predicted secondary structures of hairpin loops derived from ribozymes (c1, c2, c3) in either the presence or absence of m7G. Oligo (+) and (−) indicate the presence and absence of m7G, respectively. Sequences complementary to m7G are shown in red. Dotted lines indicate base pairs between ribozymes and m7G.

when these ribozymes are activated by binding with the oligonucleotide ligand, a substitute for helix 4 seems to be needed to stabilize the active structure of loop B. Sequence analyses of these clones revealed that there are complementary bases at both the 5′- and 3′-sides of loop C, and these are shown in Table 1 (underlined bases). If the G•U wobble base pair is included, then 2∼6 bases at the 5′- and 3′-sides of loop C are complementary with each other. Therefore, we think that the complementary bases at the joint of loop C became a pseudo-helix 4 in binding with the RLO. The complementary sequences with m7G follow the pseudo-helix 4, although some bases are inserted between them, as shown in Table 1.

Analysis of Self-Cleavage Activity. The cis-cleavage activities of all of the clones were assayed, by measuring the observed rate constants in either the presence or absence of m7G (Table 1). Every clone had little activity without m7G, but became active with an excess amount of m7G. Clones c1, c2, and c3 showed high observed rate constants in the presence of m7G ($k_{obs(+)}$). The $k_{obs(+)}$ values of these ribozymes were over 0.6 min^{-1} with the addition of the ligand, and especially both c2 and c3, which are deletion mutants, showed more than 300-fold activation efficiencies. The $k_{obs(+)}$ values of c4∼c8 were about 0.5 min^{-1}, and these ribozymes also showed about 200-fold activation efficiencies ($k_{obs(+)}/k_{obs(-)}$). Other ribozymes (c9∼c17) were also active in the presence of m7G; however, their $k_{obs(+)}$ values were below 0.4 min^{-1}.

Clones c1∼c8, which showed $k_{obs(+)}$ values over 0.5 min^{-1}, contained seven bases complementary with m7G, as shown in Table 1, and both the 5′- and 3′-sides of loop C are complementary to form the pseudo-helix 4. On the other hand, the ribozymes (c9∼c17), which exhibited low activities, do not have more than six bases that are complementary with m7G, and furthermore, they lack the complement at both ends of loop C.

Clones, c1, c2, and c3 indicated high cleavage activities among all of the clones, and the predicted secondary structures with and without m7G are shown in Figure 3. Since the active structure of loop B should be similar to that of the wild-type hairpin ribozyme (Figure 3a), it is thought that these ribozymes form the structures shown in Figure 3b in the presence of m7G. In c1, which was found most frequently, four base pairs are possible at the pseudo-helix 4 (base numbers 1∼4/17∼20 in loop C) of loop C. In c2, three Watson-Click base pairs and one G•U wobble pair are formed, and in c3, three Watson-Click base pairs can be formed at the joint of loop C (base number 1∼3/18∼20 in loop C). In these ribozymes, the binding sequences with m7G are adjacent to pseudo-helix 4, and there are one or two bases between the binding sequences with m7G and the pseudo-helix 4. The active structures formed between the ribozymes and the regulator oligonucleotide are pseudo-half-knot structures. When a complementary oligonucleotide hybridizes with a hairpin loop, one-half of the pseudoknot structure is formed between the loop and the oligonucleotide, which is called a pseudo-half-knot structure (*31*, *32*). The pseudo-half-knot also involves a single-stranded loop region, and the bases from G5 to U8 of the c1-loop seem to correspond to the single-stranded loop, although U8 may form a wobble base pair with the 3′-end guanosine of m7G.

Since these ribozymes are inactive without m7G, it is thought that the loop C structures of the inactive form differ from the active forms. We predicted the secondary structures of the entire domain II region in the absence of m7G by using the mfold program (*33*), and the structures of loop C, including four bases (C25, A26, G36, U37) in loop B, are shown in Figure 3b as oligo (−) structures. Interestingly, every loop C of these ribozymes folds into a stable helix−loop structure with a four-base turn, as shown in the Figure 3b oligo (−), and the sequences complementary to m7G are located at the single-stranded regions. In the absence of m7G,

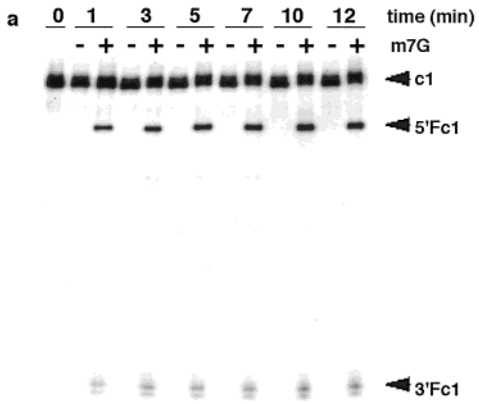
every structure has the ability to form seven connective base pairs, including one G·U wobble pair, although the G·U wobble pair of c2 is at the center of the helix. If these hairpin loops are formed without the regulator oligonucleotide, then these ribozymes should be inactive due to the disruption of the active loop B conformation.

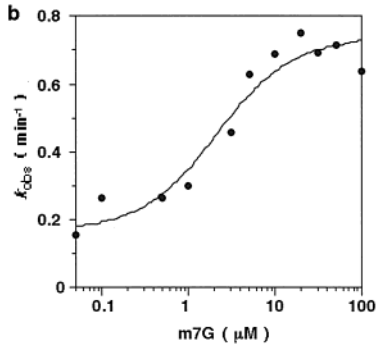
The location of the sequences complementary to m7G might facilitate their contact with m7G, and the binding with m7G might promote slippage of the base pairs of loop C. This slippage may be allowed, because two regions complementary to 3′-side of the loop C are located tandemly at the 5′-side of loop C. It is thought that after m7G binding, the newly formed helix with m7G connectively stacks with the pseudo-helix 4. Although the number of intramolecular base pairs within loop C was decreased, the stacking effect might compensate for the instability. Therefore, the loop C structure of the active form may not be unstable. A similar slip mechanism has also been reported in the FMN-binding hammerhead ribozyme (*4*); however, the structure is different from that obtained with this oligonucleotide-binding ribozyme. It is noteworthy that the m7G binding sequence is located at the 3′-side of loop C in each ribozyme (c1, c2, c3). These loop C structures binding with the oligonucleotide correspond to pseudo-half-knot structures, which mean one-half of a pseudoknot structure. It is known that the pseudoknot structure contains two single-stranded loop regions (L1 and L2), which have different lengths (*34*, *35*). The 5′-side loop (L1) of the pseudoknot is shorter than the 3′-side loop (L2) in the pseudoknot. The reason for the different lengths is that the L1 loop passes through the major groove, in contrast to L2 passing through minor groove. When an oligonucleotide binds with the 5′- or 3′-side of a hairpin loop, an alternative single stranded loop, corresponding to L2 or L1 of the pseudoknot, is formed. These active structures of the ribozymes (c1, c2, c3) correspond to the L1 loop, because the oligonucleotide binds with the 3′-side of loop C. The number of bases in loop C is 20 (19 for c2 and c3), and these bases are located in the oligonucleotide binding region, the single-stranded region, and the pseudo-helix 4 region. Since L1 has a short length, the binding sequence with m7G might be located near the 3′-side of loop C in these ribozymes.

Cleavage Activity of c1. The observed rate constants shown in Table 1 were obtained under subsaturating conditions. Then, we investigated both the dissociation constant of m7G and the chemical step of the c1/m7G complex by varying the m7G concentration. The observed rate constants for the c1/m7G complex increased as the concentration of m7G increased. The rate constant of the chemical step ($k_{RLO(+)}$) and the dissociation constant (K_d) were calculated from the observed rate constants. The highest chemical step ($k_{RLO(+)}$) in the presence of a saturating amount of m7G was 0.74 min^{-1} (Figure 4b). The dissociation constant and the activation efficiency ($k_{RLO(+)}/k_{RLO(-)}$) were 2.1 μM and 224-fold, respectively. Although the chemical step was high, the cleavage rate constant was comparable to that which we had previously observed using a hairpin ribozyme (*36*).

Although the 3′-end guanosine (rG) of m7G is able to form a G·U wobble base pair with the U8 of c1, the activation of c1 was investigated with other regulator oligonucleotides (m7, r7G, r7A, d7G, and Mis2; Figure 5a). These are 2′-hydroxyl (r), 2′-deoxy (d), and 2′-*O*-methyl (m) oligonucleotides consisting of seven or eight bases. M7 is a seven-base oligonucleotide (2′-OMe(GAGUGA)rG), and r7G and d7G are eight-base oligoribo- and oligodeoxyribonucleotides, respectively, which contain the same sequence as m7G. The only exception is that the 3′-end adenosine of r7A is different from r7G. Mis2 (r(GAGUGUCG)) contains two mismatched bases (underlined). The observed rate constants of c1 self-cleavage were measured in the presence of an excess amount of these oligonucleotides.

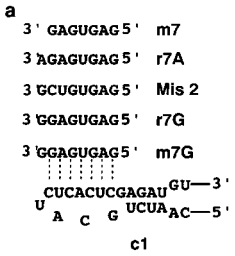
Neither d7G nor Mis2 activated c1 while both r7G and r7A activated it to a similar extent as m7G (Figure 5b). r7A yielded the slightly higher activation efficiency of c1 than





$k_{RLO(+)}$ (min^{-1})	$k_{RLO(+)}/k_{RLO(-)}$	Kd (μM)
0.74	224	2.1

FIGURE 4: (a) Analysis of the cleavage products of c1 in either the absence (−) or presence (+) of m7G. The concentrations of c1 and m7G were 1 and 50 μM, respectively. 5′Fc1 and 3′Fc1 indicate the 5′- and 3′-side fragments produced by the self-cleavage of c1. (b) Plots of the observed rate constants versus various concentrations of m7G.



b

RLO	k_{obs} (min^{-1})	End point (%)
RLO (-)	N.D.	N.D.
m7G	0.69	63
r7G	0.61	59
Mis2	N.D.	N.D.
r7A	0.73	57
m7	0.40	60

N.D. = not detected

FIGURE 5: Sequence specific activation of c1 with various RLOs. (a) The sequences of the regulator oligonucleotides (RLO) and the hairpin loop of c1. (b) Observed rate constants of c1/RLO complexes. (r) and (m) indicate ribo and 2′-*O*-methylribo. Mis 2 contains two different bases (underlined) from r7G. RLO(−) indicates the cleavage reaction without any RLO. End points indicate percentages of active molecules.

Table 2: Self-Cleavage Rate Constants (k_{cis}) of c1, c2, and c3 during Transcription Reaction[a]

	RLO(−)	r7G	r7A	m7G
	k_{cis} (min^{-1})	k_{cis} (min^{-1})	k_{cis} (min^{-1})	k_{cis} (min^{-1})
c1	N.D.	0.57 ± 0.015 (10 ± 0.76%)	0.78 ± 0.028(9 ± 0.88%)	0.57 ± 0.016 (15 ± 0.76%)
c2	N.D.	0.37 ± 0.010 (18 ± 0.88%)	0.44 ± 0.008(15 ± 0.56%)	0.46 ± 0.013 (20 ± 0.81%)
c3	N.D.	0.56 ± 0.020 (13 ± 0.99%)	0.57 ± 0.022 (10 ± 1.1%)	0.78 ± 0.075 (21 ± 2.0%)
WT	0.74 ± 0.048 (33 ± 1.1%)			

[a] Percentages of remaining full-length ribozyme are shown in parentheses. N. D. = not detected. WT indicates the wild type hairpin ribozyme.

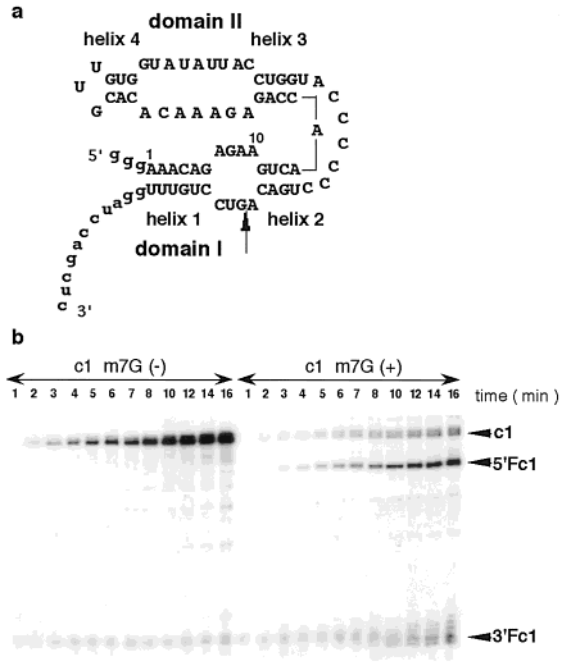


FIGURE 6: (a) The secondary structure of the cis-cleavage ribozyme with the wild-type sequences. The arrow indicates the cleavage site. (b) Cleavage reactions of c1 in either the absence (−) or presence (+) of m7G. The substrate RNA is connected with a hexa cytidylate linker to show the cis-cleavage. The 5′- and 3′-side cleavage fragments are shown in 5′Fc1 and 3′Fc1.

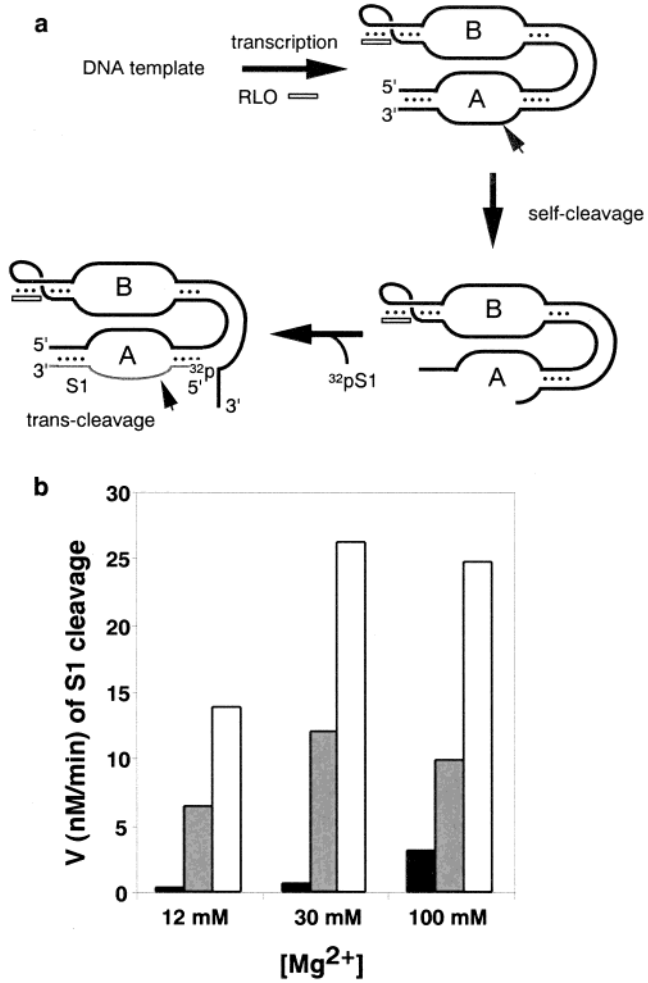


FIGURE 7: Trans-cleavage reaction catalyzed by the product from the cis-cleavage. (a) A scheme of the trans-cleavage. The ribozyme is shown by a schematic drawing. Both A and B indicate internal loops, and arrows are cleavage sites. The S1, which is an exogenous substrate, and the RLO are indicated by a gray line and a white bar, respectively. (b) Initial velocities of S1-cleavage for each magnesium ion concentration. Black, gray, and white bars indicate the concentrations of m7G in the trans-cleavage reactions. Black bars, without m7G; gray bars, [m7G] = 0.04 μM; white bars, [m7G] = 0.08 μM. The concentration of S1 was 0.8 μM in the trans-cleavage reaction.

the other oligonucleotides. The reason for this result could be that the 3′-end adenosine of r7A might form a base pair with U8 of c1, although the cleavage rate constants are almost the same. M7 also promoted the cleavage of c1, and the cleavage activity of the c1/m7 complex was slightly lower than that of c1/m7G. Furthermore, Mis2, which forms two mismatched base pairs with c1, did not induce the activity of c1. Although the reaction was carried out in the presence of a subsaturating amount of the ligand, these results suggest that the instability between the ligand and the ribozyme might decrease the activation of the ribozyme.

Self-Cleavage in Transcription. The cis-cleavage activities of the allosteric hairpin ribozymes were investigated in the above chapters using isolated RNA transcripts. Next, we compared the self-cleavage activities of c1, c2, and c3 with that of the wild-type hairpin ribozyme. We constructed the wild-type hairpin ribozyme leading to the cis-cleavage reaction (Figure 6a). Since the cleavage activity of the wild-type hairpin ribozyme cannot be controlled, we measured the self-cleavage activities during the transcription. M7G, r7G, and r7A (Figure 5a) were used as the RLOs.

In the cleavage reactions, c1, c2, and c3 did not show self-cleavage without the RLO; however, in the presence of 30 μM of RLO, these ribozymes allowed self-cleavage during the transcriptions. Since an excess amount of the RLO over the template DNA was added, we applied the same analysis method to the *cis*-cleavage of the allosteric ribozymes as the wild type, and the *cis*-cleavage rate constants (k_{cis}) during the transcriptions were calculated (Table 2). The cleavage reactions revealed that the k_{cis} values of the c1/m7G and c2/m7G complexes were slightly lower than that of the wild type. On the other hand, the c3/m7G complex, which was the one base deletion mutant of c1, showed almost the same k_{cis} value as the wild type.

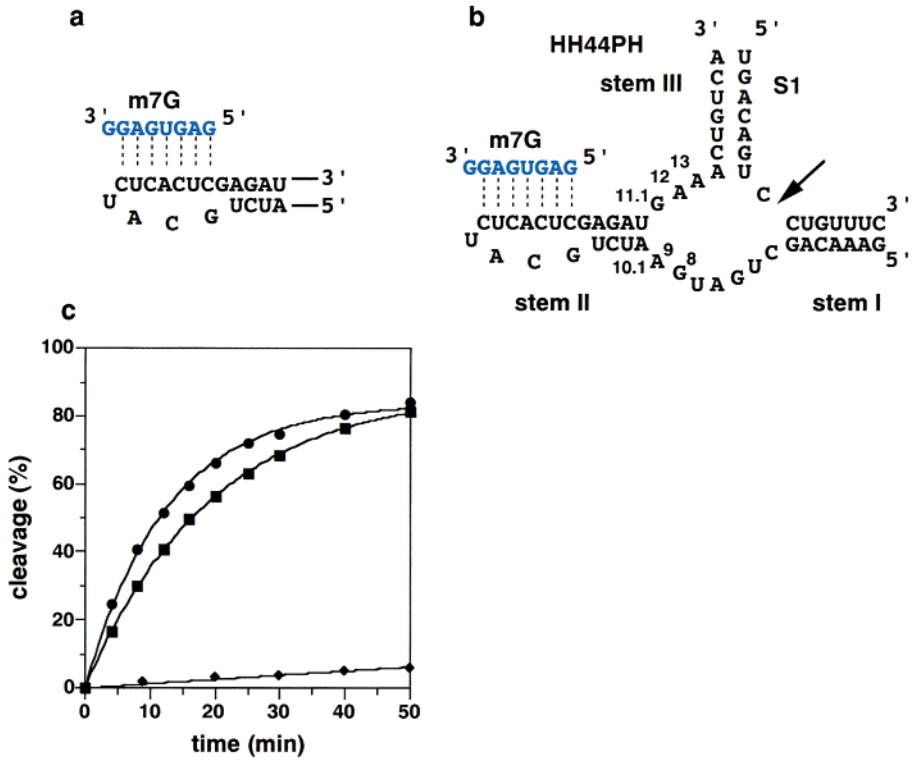


FIGURE 8: Construction of a hammerhead ribozyme with a c1 loop. (a) Hairpin loop derived from c1. (b) Secondary structure of the hammerhead ribozyme containing the c1 loop in the presence of m7G. (c) Plots of cleavage percent versus reaction time. Solid circles, (HH44PH/m7G); solid squares, (HH44PH/r7G); solid diamonds, (HH44PH without RLO).

The c1, c2, and c3 ribozymes also showed *cis*-cleavage during their transcription reactions in the presence of either r7G or r7A. The same extent of cleavage was promoted by r7G in either c1 or c3, while the activity of the c2/r7G complex was slightly lower than that of these ribozymes. The r7A oligonucleotide activated c1 most efficiently, and this result is consistent with the result from the cleavage reaction, which used the isolated c1 transcript as described above (Figure 5b). When r7A binds with c1 or c3, the 3′-end adenosine of r7A is able to form an A·U base pair with U8; however, c3 is shorter than c1, by one nucleotide, in the pseudo-helix 4 region. Therefore, it is thought that the single-stranded region of the c3-loop might be stretched more than that of c1. Even though r7A bound with c3, the U8 of c3 might have difficulty forming an A·U base pair, due to the stretched single-stranded loop region. On the other hand, U8 of c1 might adopt a structure that allows an A·U base pair, because the single-stranded loop region is not so tight. Since U8 of c3 would have difficulty forming an A·U base pair, c3 might indicate almost the same cleavage activity in the presence of either r7A or r7G. However, c3 was cleaved efficiently with m7G. This is probably because the m7G binding was stabilized by the 2′-*O*-methyl groups.

The wild type remained 33% of the total ribozyme as uncleaved transcripts; however, the percentages of uncleaved ribozymes for c1, c2, and c3 were lower than that of the wild type. The high remaining percentage for the wild type ribozyme might be derived from the ligation activity. The hairpin ribozyme possesses ligation activity in addition to cleavage activity, and the *cis*-ligation proceeds more efficiently than the trans-ligation.

The c1 ribozyme showed self-cleavage activity with an excess amount of RLO. Since the *cis*-cleavage is an intramolecular reaction, we investigated whether the 5′-side cleavage product (5′Fc1) derived from the self-cleavage could catalyze an intermolecular cleavage reaction. After the *cis*-cleavage reaction was carried out, during transcription in the presence of small amount of RLO, a ^{32}P labeled substrate RNA (S1, Figure 1a), which had been used for the investigation of *trans*-cleavage of the wild hairpin ribozyme, was added to the reaction solution. The scheme of the reaction is shown in Figure 7a. The *cis*-cleavage reaction was performed for 1 h by using the standard method as described above, and then the *trans*-cleavage was carried out with different magnesium ion concentrations. The percentage of cleavage was measured at each time point, and the initial velocities of S1-cleavage were obtained (Figure 7b). As the result of the cleavage reaction, the transcription solutions containing m7G resulted in the trans cleavage of S1, and the cleavage activity was dependent on the concentration of m7G. The *trans* cleavage was efficient in the presence of higher magnesium ion concentrations. These results indicate that the self-cleavage products could also function as a catalyst for *trans*-cleavage. Although the concentration of m7G was much lower than that used in the measurement of the k_{cis} values described above, the 5′Fc1/m7G complex cleaved S1, which existed at higher concentrations than m7G. This result suggests that c1 could transmit the signal of m7G binding to the cleavage of S1 with amplification of the signal.

Activation of the Hammerhead Ribozyme Involving the c1 Loop. The activation hairpin module of c1 seems to be changed by responding to the presence of the regulator oligonucleotides, as shown in Figure 8. To investigate whether the module of c1 can be applied to the control of another ribozyme, we introduced it into stem II of a hammerhead ribozyme. The modified hammerhead ribozyme

(HH44PH) consists of 44 bases and is designed to cleave a substrate RNA (S1) *in trans*. If the structural change of c1 is promoted by the binding of m7G, as shown in Figure 8, then HH44PH would become active by the addition of m7G, and would cleave the substrate RNA.

We carried out the cleavage reaction of the target RNA by HH44PH under single turnover conditions using excess amount of m7G or r7G, and measured the observed rate constants of HH44PH. HH44PH showed little cleavage activity in the absence of m7G, while on the other hand, in the presence of either m7G or r7G, the HH44PH/RLO complexes cleaved the substrate RNA at the expected site. The observed rate constants of the HH44PH, HH44PH/m7G, and HH44PH/r7G complexes were 0.0013, 0.080, and 0.052 min^{-1}, respectively. These results indicate that the loop motif of c1 can be applied to the allosteric control of other functional RNAs. It is known that $G_{10.1}$·$C_{11.1}$ is preferred over the $A_{10.1}$·$U_{11.1}$ base pair at the joint of stem II in the hammerhead ribozyme (*37*). Since the base pair in HH44PH is $A_{10.1}$·$U_{11.1}$, the activity of HH44PH was slightly lower than the wild type ribozyme previously reported (*13*). The activation of HH44PH shows that the hairpin module of c1 adopts the expected active form, as shown in Figure 8. We also carried out cleavage reactions under excess substrate condition (data not shown). The hammerhead ribozyme indicated cleavage activity, even though more substrate than the oligonucleotide was present in the reaction solution.

CONCLUSION

We carried out *in vitro* selection to construct an allosteric hairpin ribozyme, which is activated by the addition of a short oligonucleotide (RLO). A random domain was inserted in the hairpin loop region of the wild type ribozyme. After the selection, we isolated ribozymes that showed the self-cleavage activities only in the presence of the RLO. The allosteric hairpin ribozymes had the characteristic hairpin loop sequences, in which the base pairs slip by binding with the RLO. The module structure transmitted the structural change to the core of the ribozyme, and controlled the active or inactive structure of the ribozyme. In the absence of the RLO, the sequences complementary to the RLO are in a single-stranded region, which allows the RLO to bind with the module easily.

The induction of the cleavage is sequence specific, and the affinity between the ribozyme and the RLO is high, due to the formation of base pairs. The ribozymes might be effective for *in vivo* analyses of antisense activities, if the ribozyme is activated by the antisense oligonucleotide.

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