Effect of metal ions and sequence of deoxyribozymes on their RNA cleavage activity

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Catalytic DNA is a promising class of nucleic acid enzyme for possible use as a therapeutic agent and is also interesting in comparison with catalytic RNAs. In this study, we investigated the effect of metal ions and sequence on an original deoxyribozyme, d(GCCTGGCAG$_1$G$_2$C$_3$T$_4$A$_5$G$_6$C$_7$T$_8$A$_9$C$_{10}$A$_{11}$A$_{12}$C$_{13}$G$_{14}$A$_{15}$GTCCCT), which binds to an RNA substrate, r(AGGGACA↓UGCCAGGC), cleaving the RNA substrate at one site indicated by the arrow. The results show that the ability of metal ions to promote the RNA cleavage reaction by the original deoxyribozyme is $Mn^{2+} > Mg^{2+} > Ca^{2+} \gg Ba^{2+}$. This result is very similar to the previous tendency observed in the case of a hammerhead ribozyme. Thus, these results suggest that RNA cleavage by the deoxyribozyme dependence on metal ions for catalysis is similar to that with ribozymes. On the other hand, a nucleotide deletion from the active domain of the original deoxyribozyme results in a novel deoxyribozyme with high Ca^{2+}-dependency, a situation which is not observed with the original deoxyribozyme or hammerhead ribozymes.

Introduction

Catalytic RNAs have the potential to produce reactive structures through self-assembly. Their catalytic reactions depend on their structures.[1,2] Recent NMR and X-ray studies showed the specific RNA–RNA interactions and RNA–metal interactions that are important for their functions.[3–9] These studies also demonstrate the importance of hairpin loops and unpaired regions such as bulges and internal loops. Mismatches like G·A and G·U in internal loops and GAAA tetraloops in hairpin structures are especially interesting structures, because they play roles as metal ion binding sites, or receptor–acceptors in the tertiary structure. Earlier thermodynamic studies showed that mismatches such as G·A, G·U, and GAAA tetraloops form stable structures.[10–13] Thus, both structural and thermodynamic studies show that GAAA tetraloops and the unpaired regions such as G·A and G·U are specific conformations. Such studies of RNA function are useful for understanding the well known ribozymes such as group I and II introns,[14,15] ribonuclease P,[16] HDV ribozymes,[17] hammerhead ribozymes,[18] hairpin ribozymes,[19] and leadzymes.[20,21] They would also be useful in the design of new ribozyme systems. In the case of hammerhead ribozymes, predictions of reaction efficiency have been reported using thermodynamic data based on the nearest-neighbor parameters.[22] In addition, the activities of ribozymes depend on metal ions that play roles in folding and as cofactors during catalysis. In order to understand the detailed roles of metal ions in RNA cleavage mechanisms, the effect of different metal ions on the ribozyme reaction has been investigated.[23–28] Such results are also useful in the design of new ribozyme systems, because ribozyme reactions depend on the unique properties of metal ions.

In vitro selection methods have produced specific DNAs with self-cleavage,[29,30] RNA cleavage,[31,32] or metallation[33,34] activities. Their secondary structures contain unpaired regions like those seen in catalytic RNAs. A deoxyribozyme with RNA cleavage activity also has an unpaired region as shown in Fig. 1. Furthermore, this particular deoxyribozyme has high RNA cleavage activity for target RNA sequences of HIV-1 *gag*/*pol*, *env*, *vpr*, *tat*, and *nef* mRNAs, and *BCR-ABL* chimeric L6 mRNA in the presence of Mg^{2+}.[32,35,36] This deoxyribozyme might have more uses if more detailed information was available, because the stability of DNA/RNA hybrids within the stem regions of the deoxyribozyme–RNA substrate complex may be predicted by using thermodynamic parameters based on nearest-neighbor models as was done for the case of hammerhead ribozymes.[37]

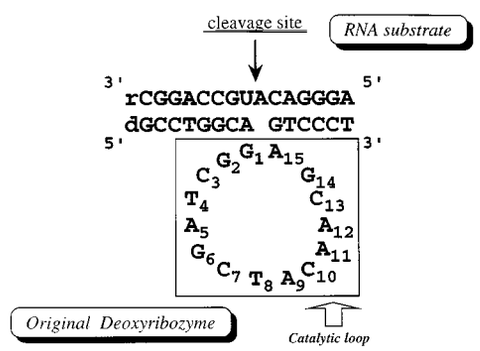

Fig. 1 Secondary structure of the original deoxyribozyme and the RNA substrate complex. The arrow indicates the cleavage site.

In this paper, we investigated the effect of metal ions and sequence in the catalytic loop of the deoxyribozyme on its RNA cleavage activity. The results give useful information for improving and designing deoxyribozymes as well as ribozymes. The results also show that the ability of metal ions to promote this RNA cleavage reaction is very similar to that observed with the hammerhead ribozymes. A nucleotide deletion from the active domain of the original deoxyribozyme results in a novel deoxyribozyme with much higher Ca^{2+}-requirement for the RNA cleavage than was seen for the original deoxyribozymes or hammerhead ribozymes.

Experimental

Preparation of oligonucleotides

DNA and RNA oligonucleotides were synthesized on solid supports using the phosphoramidite method on an Applied Biosystems model 391 DNA/RNA synthesizer.[37] DNA oligonucleotides with abasic nucleotides were also similarly syn-

thesized using a dSpacer CE Phosphoramidite (Glen Research Co., Ltd.). The synthesized oligodeoxyribonucleotides were deprotected by treatment with concentrated 25% ammonia at 55 °C for 3 h. After drying under vacuum, the oligodeoxyribonucleotides were passed through a Poly-Pak cartridge (Glen Research Co., Ltd.) with 2% TFA to remove dimethoxytrityl groups. The synthesized oligoribonucleotides were removed from the solid support and the base-blocking groups were removed by treatment with concentrated 25% ammonia in ethanol (3 : 1, v/v) at 55 °C for 3 h.[38] After drying in vacuum, the 2′-silyl protection groups were removed by resuspending the pellet in 50 equivalents of 1.0 M tetrabutylammonium fluoride (TBAF) per equivalent of silyl, and the mixtures were incubated overnight in the dark at room temperature.[38] The oligoribonucleotides were then passed through a C-18 Sep-Pak cartridge column (Waters) for desalting. After deblocking, the DNA and RNA oligonucleotides were purified by electrophoresis on 20% polyacrylamide (19% acrylamide and 1% bisacrylamide)–7 M urea denaturing gels. Final purities of the RNA and DNA oligonucleotides were determined to be greater than 99% by HPLC on a TSKgel OligoDNA RP column (Tosoh) with a linear gradient of 0–50% methanol–H_2O containing 0.1 M TEAA (pH 7.0). The oligonucleotides were desalted again with a C18 Sep-Pak cartridge column. Single-strand concentrations of purified DNA and RNA oligonucleotide were determined by measuring the absorbance at 260 or 280 nm at high temperature with a Hitachi U-3210 spectrophotometer connected to a Hitachi SPR-10 thermoprogrammer as described previously.[37] Single-strand extinction coefficients were calculated from mononucleotide and dinucleotide data by using a nearest-neighbor approximation.[39] The RNA substrate with a 5′-OH was 5′-end labeled in 25 μL reaction mixture containing 25 pM RNA, 150 μCi [γ-^{32}P] ATP (6000 Ci mol^{-1}, New England Nuclear), 2.5 μL 10 × kinase buffer (500 mM Tris–HCl (pH 7.6), 100 mM $MgCl_2$, and 100 mM 2-mercaptoethanol), and 10 units T4 polynucleotide kinase (Toyobo Co., Ltd.).

Cleavage reactions

RNA cleavage reactions by deoxyribozymes were performed under single-turnover conditions. Experiments with the deoxyribozyme in excess over the RNA substrate were carried out in 25 mM divalent metal ion and 50 mM Tris–HCl (pH 8.0) at 37 °C. The ^{32}P-labeled RNA substrate (5 nM) and 1 μM deoxyribozyme were heated together to 90 °C for 3 min, cooled slowly, and incubated at 37 °C for 30 min. All cleavage reactions were initiated by the addition of the buffer solution containing divalent metal ions at 37 °C. The cleavage reactions were terminated by removing aliquots from the reaction mixture at appropriate intervals and mixing them with an equal volume of 100 mM Na_2EDTA, 9 M urea, 0.02% bromophenol blue, and 0.02% xylene cyanol. The labeled product and substrate were separated by electrophoresis on 20% polyacrylamide (19% acrylamide and 1% bisacrylamide)–7 M urea denaturing gels. The RNA cleavage yields were determined by quantitation of radioactivity in the bands of labeled products and substrate with a Bio-Image Analyzer model BAS 2000 (Fuji Film, Tokyo). Multiple-turnover experiments with the RNA substrate in 10-fold excess over the deoxyribozyme were carried out in the buffer solution at 37 °C. Initial rates corresponding to the first 15% of reaction were used to obtain rate constants, and the k_{cat} and K_m values were calculated from Eadie–Hofstee plots.[40]

Results

Effect of divalent metal ions on RNA cleavage activity by original deoxyribozyme

The secondary structures of the complex of the original deoxyribozyme [d(GCCTGGCAG$_1$G$_2$C$_3$T$_4$A$_5$G$_6$C$_7$T$_8$A$_9$C$_{10}$A$_{11}$A$_{12}$-C$_{13}$G$_{14}$A$_{15}$GTCCCT)] and the RNA substrate [r(AGGG-ACA↓UGCCAGGC)] are shown in Fig. 1. The cleavage site is between 5′-rApU-3′ in the asymmetric internal loop, which is indicated by an arrow in Fig. 1. The original deoxyribozyme requires Mg^{2+} for RNA cleavage.[32] To investigate the effect of other metal ions on the RNA cleavage, the cleavage reactions were carried out in the presence of various divalent metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ba^{2+}, and Pb^{2+}). Fig. 2 shows the result of the RNA cleavage reaction by the original deoxyribozyme in the presence of 25 mM divalent metal ions at 37 °C after 30 min incubation. One cleaved band was observed in the presence of Mn^{2+}, Ca^{2+}, or Mg^{2+}. The migrations of the bands were the same in all cases. On the other hand, a few product bands were observed in the presence of Pb^{2+}. This RNA cleavage reaction in the presence of Pb^{2+} was not site specific cleavage. This cleavage was observed without the original deoxyribozyme. Thus, non-specific cleavage may be due to the extensive degradation of RNA by high Pb^{2+} concentrations.[2] In the presence of Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Ba^{2+}, no bands were observed after 30 min incubation. However, a cleavage band was observed after 180 min incubation in the presence of Ba^{2+}. Furthermore, RNA cleavage was not observed in the presence of Mg^{2+}, Mn^{2+}, or Ca^{2+} in the absence of the deoxyribozyme. Thus, this original deoxyribozyme efficiently catalyzes the site specific RNA cleavage in the presence of Mn^{2+}, Ca^{2+}, or Mg^{2+}.

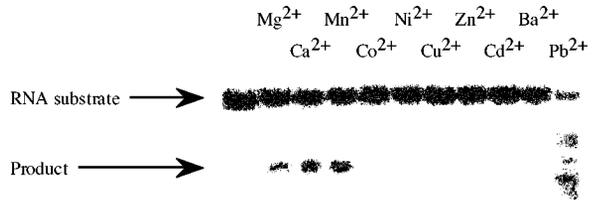

Fig. 2 Autoradiogram of denaturing 20% polyacrylamide gel showing the cleavage of 5 nM 5′-end labeled RNA substrate by 1 μM original deoxyribozyme in a buffer containing 50 mM Tris–HCl (pH 8.0) and 25 mM divalent metal ions after 30 min incubation at 37 °C.

Nucleotide deletion within the catalytic loop of the deoxyribozyme

This catalytic loop with the original deoxyribozyme has a large loop like the hammerhead ribozyme. In the case of the hammerhead ribozyme, the deletion of stem-loop II that has little effect on the cleavage activity led to cleavage of a substrate at two independent sites.[41] The mutant leadzyme with the deletion of one nucleotide from the active domain showed higher activity than the original leadzyme.[42] Thus, the deletion of nucleotides from the active domain showed unique and different properties. What is the effect of the nucleotide deletion when it occurs in the deoxyribozyme system?

Fig. 3a shows the sequences of the original and ML **1**–**11** deoxyribozymes that had some nucleotides removed from the loop domain used. This original sequence was selected in the presence of Mg^{2+}, suggesting that mismatches within the catalytic core may act as a good binding pocket for a metal ion such as is seen with many ribozymes. Thermodynamic studies of DNA mismatches show that G·A and G·T mismatches are favorable, although their detailed stabilities depend on the closing base pairs.[43,44] So, each mutant deoxyribozyme is designed for the much shorter sequence within the catalytic loop to form some mismatches such as G·A and G·T. Fig. 3b shows the RNA cleavage yields by the original and mutant variant deoxyribozymes in the presence of 25 mM Ca^{2+}, Mg^{2+}, or Mn^{2+} after 90 min incubation. The RNA cleavage site of each mutant deoxyribozyme was between 5′-rApU-3′ in the asymmetric internal loop as was the case with the original deoxyribozyme. The cleavage activities of many

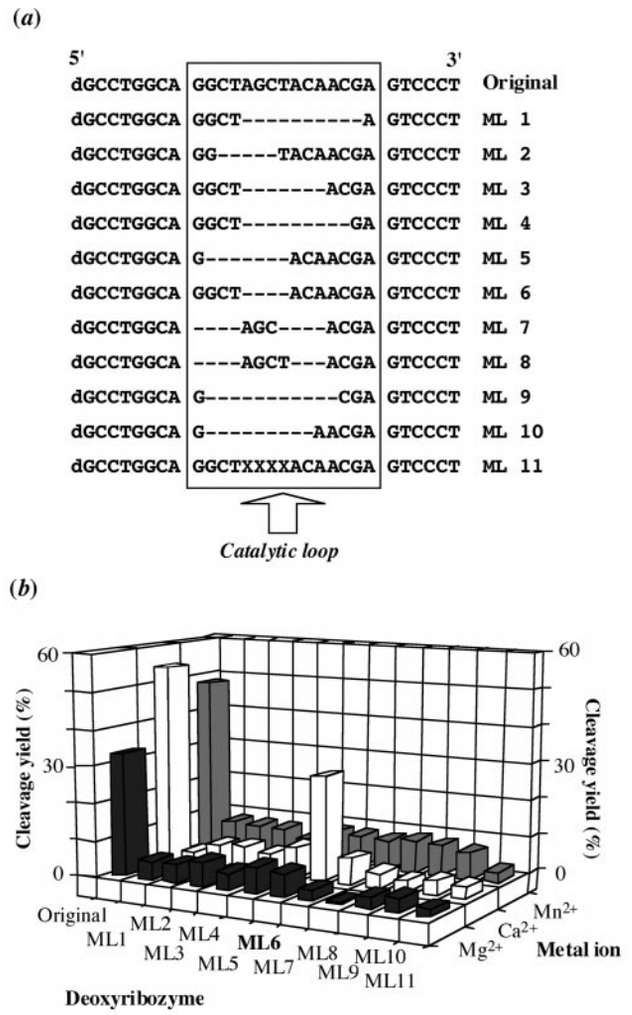

Fig. 3 (a) Sequences of the original and ML **1**–**11** deoxyribozymes. X denotes an abasic nucleotide. (b) Three dimensional histograms of RNA cleavage yields. All experiments were done under single-turnover conditions with 1 μM original and ML **1**–**11** deoxyribozymes and 5 nM 5′-end labeled RNA substrate after 90 min incubation at 37 °C. The buffer was 50 mM Tris–HCl (pH 8.0) containing 25 mM Ca^{2+}, Mg^{2+}, or Mn^{2+}.

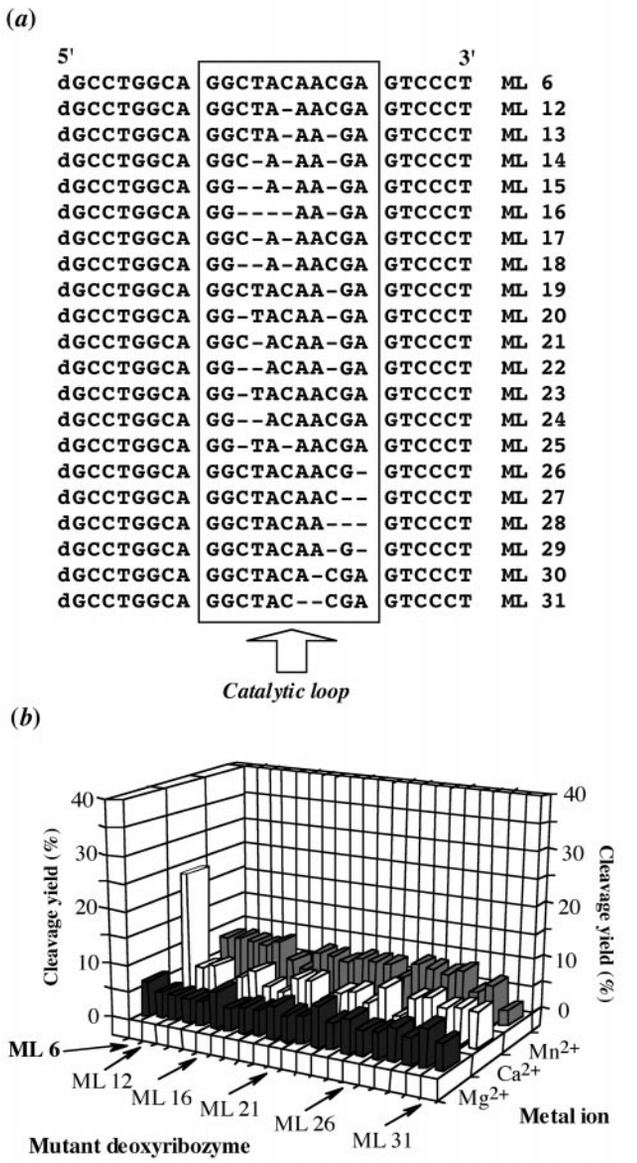

Fig. 4 (a) Sequences of the ML **12**–**31** deoxyribozymes. (b) Three dimensional histograms of RNA cleavage yields. All experiments were done under single-turnover conditions with 1 μM mutant deoxyribozymes and 5 nM 5′-end labeled RNA substrate after 90 min incubation at 37 °C. The buffer was 50 mM Tris–HCl (pH 8.0) containing 25 mM Ca^{2+}, Mg^{2+}, or Mn^{2+}.

mutant deoxyribozymes were less than 10% as shown in Fig. 3b. However, the cleavage yield by ML **6** deoxyribozyme [d(GCCTGGCAG$_1$G$_2$C$_3$T$_4$A$_9$C$_{10}$A$_{11}$A$_{12}$C$_{13}$G$_{14}$A$_{15}$GTCCCT)] after 90 min incubation in the presence of 25 mM Ca^{2+} was 22.2%. The catalytic loop of the ML **6** deoxyribozyme, which consists of 11 deoxyribonucleotides, has had d($A_5G_6C_7T_8$) deleted from the original deoxyribozyme. To check whether ML **6** deoxyribozyme is a specific or special deoxyribozyme consisting of only conserved nucleotides of the original deoxyribozyme, ML **11** deoxyribozyme which had d($A_5G_6C_7T_8$) replaced by abasic nucleotides was tested. ML **11** deoxyribozyme showed no significant cleavage activity in the presence of Mg^{2+}, Mn^{2+}, or Ca^{2+}.

To investigate further the minimum nucleotides required within the catalytic loop for site-specific RNA cleavage, the effect of nucleotide deletions in the loop region of the ML **6** deoxyribozyme were studied. Fig. 4a shows the sequence of the ML **6** and ML **12**–**31** deoxyribozymes, which have some nucleotides deleted from the ML **6** deoxyribozyme. Fig. 4b shows the RNA cleavage yields by the ML **6** and ML **12**–**31** deoxyribozymes in the presence of 25 mM Ca^{2+}, Mg^{2+}, or Mn^{2+} after 90 min incubation. RNA cleavage yields by ML **12**–**31** deoxyribozymes were less than 10%, although the RNA cleavage sites are between 5′-rApU-3′ in the asymmetric internal loop (data not shown). Thus, the ML **6** deoxyribozyme has a much shorter active domain and a new metal-ion dependency that depends on both the sequence and the number of residues within the catalytic loop.

Kinetic effect of metal ions and nucleotide sequence of deoxyribozymes on RNA cleavage

To clarify the effect of metal ions and nucleotide deletions on the RNA cleavage, we measured the kinetic parameters for the RNA cleavage reaction under multiple-turnover conditions. Fig. 5 shows the time course of RNA cleavage with the original deoxyribozyme in the presence of 25 mM Mg^{2+}, Mn^{2+}, or Ca^{2+}. Burst kinetics were not observed in any reaction, suggesting that the rate-limiting step is not a product-release step.[45] In the case of the original deoxyribozymes, the nature of the metal ions contributes to both k_{cat} and K_m values as shown in Table 1. These results indicate that the contributions of each metal ion to the RNA cleavage step and the deoxyribozyme–RNA substrate binding step are different. The apparent second-order rate constants (k_{cat}/K_m values) of Mn^{2+}, Mg^{2+}, Ca^{2+}, and Ba^{2+} were 7.0×10^7, 2.2×10^7, 1.4×10^7, and 0.26×10^7 M^{-1} min^{-1},

Table 1 Kinetic parameters for RNA cleavage by original or mutant deoxyribozymes[a]

Deoxyribozyme	Metal ion	k_{cat}/min^{-1}	K_m/nm	$k_{cat}/K_m/10^7$ M^{-1} min^{-1}
Original	Mn^{2+}	1.19 ± 0.12	17.0 ± 0.89	7.0
Original	Mg^{2+}	0.961 ± 0.052	44.6 ± 1.2	2.2
Original	Ca^{2+}	0.863 ± 0.074	63.9 ± 1.6	1.4
Original	Ba^{2+}	0.101 ± 0.012	39.1 ± 3.6	0.26
ML **6**	Ca^{2+}	2.04 ± 0.92	123 ± 3.0	1.7
ML **6**	Mn^{2+}	0.316 ± 0.053	275 ± 4.4	0.11
ML **6**	Mg^{2+}	0.209 ± 0.023	291 ± 2.6	0.072
ML **11**	Ca^{2+}	0.222 ± 0.014	48.3 ± 0.64	0.46
ML **11**	Mg^{2+}	0.0994 ± 0.0076	46.0 ± 0.71	0.22
ML **11**	Mn^{2+}	0.0301 ± 0.0046	18.4 ± 0.18	0.16

[a] All experiments were done in a buffer containing 50 mM Tris–HCl (pH 8.0) and 25 mM divalent metal ion at 37 °C.

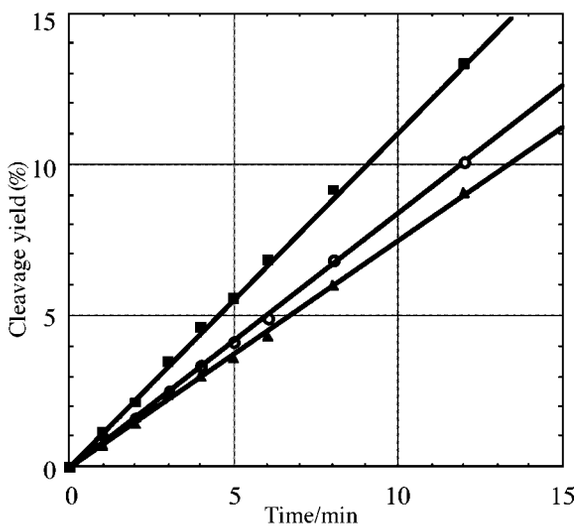

Fig. 5 Extent of RNA substrate cleavage *vs.* time by original deoxyribozyme in the presence of Mn^{2+}(■), Mg^{2+}(○), or Ca^{2+}(▲).

respectively. Thus, the order of efficiency of the ions is $Mn^{2+} > Mg^{2+} > Ca^{2+} \gg Ba^{2+}$. On the other hand, the apparent second-order rate constants of ML **6** deoxyribozyme in the presence of Mn^{2+}, Mg^{2+}, and Ca^{2+} were 0.11×10^7, 0.072×10^7, and 1.7×10^7 M^{-1} min^{-1}, respectively. Thus, the order of efficiency of the ions with ML **6** deoxyribozyme is $Ca^{2+} \gg Mn^{2+} > Mg^{2+}$. These results suggest that the ML **6** deoxyribozyme has catalytic efficiency as high as the original deoxyribozyme in the presence of Ca^{2+}. Furthermore, the difference of k_{cat}/K_m values with the ML **6** deoxyribozyme between the most active Ca^{2+} and the most inactive Mg^{2+} is about 20-fold, although that with the original deoxyribozyme is about 5-fold. In the case of ML **11** deoxyribozyme, the k_{cat}/K_m value for Ca^{2+} is 27% as compared with the ML **6** and the maximum difference between the ions is about 3-fold as shown in Table 1. We also investigated the effect of concentration of the metal ions. While the maximal k_{cat} and K_m values in the presence of Ca^{2+} with ML **6** were reached by 10 mM Ca^{2+}, those in the presence of Mg^{2+} were reached by 25 mM Mg^{2+}. These data suggest that the observed k_{cat} and K_m values at 25 mM for each metal ion with ML **6** are under conditions of maximal rate with each metal ion. Thus, these kinetic results support the fact that the ML **6** deoxyribozyme has higher Ca^{2+}-selectivity for the RNA cleavage than the original one.

Effect of an abasic nucleotide introduction of ML 6 in the presence of Ca^{2+}

To identify critical residues within the catalytic loop of the ML **6** deoxyribozyme in the presence of Ca^{2+}, the activity with ML **32**–**42** deoxyribozymes that have one natural nucleotide of the ML **6** deoxyribozyme substituted by an abasic nucleotide was tested. Fig. 6 shows the predicted secondary structures of the ML **6** and ML **32**–**42** deoxyribozymes. Table 2 shows the kinetic parameters of the ML **6** and ML **32**–**42** deoxyribozymes in the presence of 25 mM Ca^{2+} at 37 °C. The apparent second-order rate constants of ML **34**–**37** deoxyribozymes are similar to the ML **6** deoxyribozyme, but ML **32**, ML **33**, and ML **38**–**42** deoxyribozymes have low activities. These results suggest that G_1, G_2, A_{11}, A_{12}, C_{13}, G_{14}, and A_{15} in the catalytic loop of the ML **6** deoxyribozyme influence the RNA cleavage activity in the presence of Ca^{2+}. On the other hand, C_3, T_4, A_9, and C_{10} do not influence cleavage.

Discussion

The results reported here indicate that this deoxyribozyme has metal-ion dependencies for RNA cleavage similar to that of ribozymes. The experiments led to the discovery of the ML **6** deoxyribozyme that has a shorter catalytic loop, d(GGCTACAACGA), than in the original and that requires Ca^{2+} for efficient RNA cleavage.

Role of divalent metal ions on RNA cleavage activity by the deoxyribozyme

Santro and Joyce also investigated the effect of metal ions on k_{cat} values in the cleavage step, although their stem sequence and length of the original deoxyribozyme are different from ours.[46] They indicate that Mn^{2+} has the highest activity, and Mg^{2+} and Ca^{2+} have similar activities. This order is similar to that seen for our original deoxyribozyme, suggesting that the difference between metal ions of the effect on the k_{cat} of our deoxyribozyme is probably due to their effect on the cleavage step. Further, the ability of metal ions to promote the RNA cleavage reaction by the hammerhead ribozyme decreased in the order $Mn^{2+} > Mg^{2+} > Ca^{2+}$.[45] Thus, the order of metal ions for RNA cleavage by the original deoxyribozyme is similar to the case of the hammerhead ribozyme.

In addition, the difference between metal ions also contributes to K_m values as well as to k_{cat}, and the K_m value in the presence of Ca^{2+} is the largest in all cases. In the case of *Tetrahymena* ribozyme, Ca^{2+} led to an increase in the dissociation rate constant of the substrate from the ribozyme, so that ribozyme–substrate binding in the presence of Ca^{2+} becomes weaker than that with Mg^{2+}.[47,48] Thus, the difference of K_m values between metal ions may be directly due to the difference of stabilities of the deoxyribozyme–substrate complex with the different ions.

Novel reaction domain with Ca^{2+}-dependency

The nucleotide-deletion experiment revealed a novel, catalytically active Ca^{2+}-dependent motif that is smaller than the original deoxyribozyme. This Ca^{2+}-specificity of ML **6** deoxyribozyme depended on both k_{cat} and K_m. The k_{cat} value of

Table 2 Kinetic parameters for RNA cleavage by mutant deoxyribozymes[a]

Deoxyribozyme	Catalytic loop sequence[b]	k_{cat}/min^{-1}	K_m/nm	$k_{cat}/K_m/10^7$ M^{-1} min^{-1}
ML **6**	GGCTACAACGA	2.04 ± 0.92	123 ± 3.0	1.7
ML **32**	**X**GCTACAACGA	0.190 ± 0.078	193 ± 2.3	0.098
ML **33**	G**X**CTACAACGA	0.240 ± 0.074	191 ± 1.1	0.11
ML **34**	GG**X**TACAACGA	1.95 ± 0.49	147 ± 0.3	1.3
ML **35**	GGC**X**ACAACGA	1.98 ± 0.43	133 ± 1.6	1.5
ML **36**	GGCT**X**CAACGA	2.00 ± 0.21	132 ± 1.3	1.5
ML **37**	GGCTA**X**AACGA	1.89 ± 0.18	149 ± 0.4	1.3
ML **38**	GGCTAC**X**ACGA	0.250 ± 0.054	182 ± 0.99	0.14
ML **39**	GGCTACA**X**CGA	0.128 ± 0.074	196 ± 2.7	0.065
ML **40**	GGCTACAA**X**GA	0.430 ± 0.031	155 ± 1.6	0.28
ML **41**	GGCTACAAC**X**A	0.0412 ± 0.0097	219 ± 3.8	0.019
ML **42**	GGCTACAACG**X**	0.0123 ± 0.0019	279 ± 5.6	0.044

[a] All experiments were done in a buffer containing 50 mM Tris–HCl (pH 8.0) and 25 mM Ca^{2+} at 37 °C. [b] **X** denotes an abasic nucleotide.

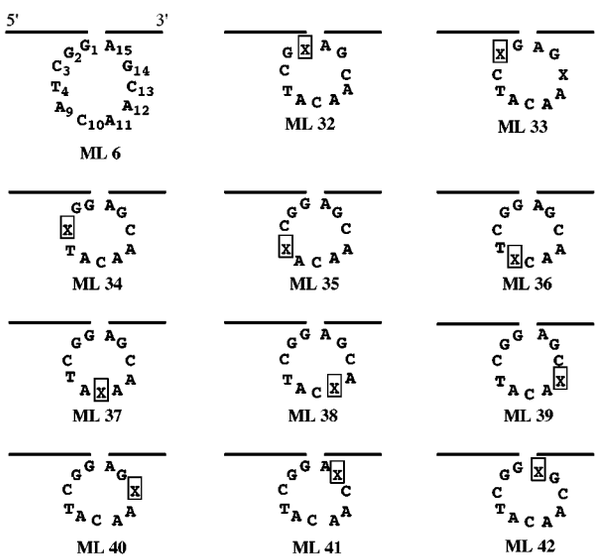

Fig. 6 Secondary structures of the ML **6** and ML **32**–**42** deoxyribozymes. X denotes an abasic nucleotide.

ML **6** deoxyribozyme in the presence of Ca^{2+} is about 10-fold larger than those of Mg^{2+} and Mn^{2+}. This value is also about 2-fold larger than that of the original deoxyribozyme. The cleavage step is sensitive to a rearrangement of bases to reach the transition state.[49] The different cation-dependencies on the k_{cat} value between the original and ML **6** deoxyribozymes would indicate that the local structure near a cleavage site of the ML **6** deoxyribozyme is significantly different from that of the original catalytic core. The cation-dependence of the ML **6** deoxyribozyme on the K_m value is also different from that of the original deoxyribozyme. Thus, Ca^{2+}-specificity may be due to the specific folding of the deoxyribozyme–RNA substrate complex that depends on the Ca^{2+}-binding site, sequence, and number of bases within the catalytic core.

Ca^{2+} has effects on the RNA folding that are different from those of Mg^{2+} and Mn^{2+}. The *Tetrahymena* ribozyme uses Mg^{2+} and Mn^{2+}, Ca^{2+} inhibits its activity.[50] This inhibition is due to not only the weaker binding of the substrate but also to the differences in the local ribozyme structure.[47,48,50,51] Ca^{2+} has relevant differences with respect to Mg^{2+}. For example, the two ions have different radii (0.99 Å for Ca^{2+} and 0.80 Å for Mg^{2+}) and different charge densities. From the point of view of the coordination patterns, the common geometry for Ca^{2+} is eight, while that for Mg^{2+} is six to seven. Thus, one might expect that Mg^{2+} and Ca^{2+} have different effects on RNA folding.[47] Lehman and Joyce obtained a *Tetrahymena* ribozyme which required only Ca^{2+} as the divalent metal ion by using *in vitro* selection.[52] Many mutations in Ca^{2+}-dependent *Tetrahymena* ribozyme were found to occur at the unpaired positions in the secondary structure, suggesting that the folding of unpaired regions would be sensitive to Mg^{2+} and Ca^{2+} differences. Thus, our finding of a new Ca^{2+}-dependent motif is reasonable, because the active domain of the deoxyribozyme is an unpaired region.

The substitution of natural nucleotides by abasic ones identifies the bases that are required for folding into a suitable conformation in the presence of Ca^{2+}. By this criterion, the G_1, G_2, A_{11}, A_{12}, C_{13}, G_{14}, and A_{15} residues in the catalytic loop of the ML **6** deoxyribozyme are important to the RNA cleavage activity in the presence of Ca^{2+}. Recently, X-ray studies indicate that a G·A mismatch is a metal ion binding site in functional RNA.[8] In the case of the ML **6** deoxyribozyme, for example, G_1·A_{12} or G_2·A_{11} base pairs could form in analogy to those in the hammerhead ribozyme. Thus, some mismatches such as G·A in the pocket consisting of G_1, G_2, A_{11}, A_{12}, C_{13}, G_{14}, and A_{15} may play a very important role in the Ca^{2+} binding. The ML **6** deoxyribozyme has a specific cation-dependency on the RNA cleavage not observed in the case of the original deoxyribozyme or the hammerhead ribozyme.

Acknowledgements

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