 

*Accounts*

# Site-Selective Activation of RNA Leading to Sequence-Selective RNA Cutters

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New types of RNA cutters for site-selective scission are prepared by combining metal ions with oligonucleotides bearing an acridine. Both lanthanide(III) ions and various divalent metal ions (e.g., Zn(II), Mn(II), and Mg(II)) are used without being bound to any sequence-recognizing moiety. The modified oligonucleotide forms a hetero-duplex with the RNA, and activates the phosphodiester linkages in front of the acridine. As a result, these linkages are preferentially hydrolyzed over the others, even though the metal ions are not fixed anywhere. The scission is efficient under physiological conditions, irrespective of the sequence at the target site. Site-selective RNA scission is also accomplished by combining an oligonucleotide bearing an acridine at its terminus, another unmodified oligonucleotide, and a metal ion. In the proposed mechanism, the acridine pushes the unpaired ribonucleotide out of the hetero-duplex and changes the conformation of RNA at the target site for the sequence-selective activation.

Site-selective RNA scission is one of the keys in the current progress of molecular biology and biotechnology.[1,2] This technique is essential for the regulation of expression of a predetermined gene for bio-regulation, advanced therapy, and other needs. Furthermore, it is useful for opening a new RNA world in which RNA must be manipulated and transformed into a desired form. However, none of the naturally occurring ribonucleases shows such a high sequence-selectivity as is required here. At present, RNA enzymes (ribozymes), discovered by Cech et al.,[3] have been widely used for these purposes.[4] However, "completely-artificial ribozyme mimics" are also attractive, since (1) they can be freely designed and synthesized as we need them and (2) they are provided with desired functions at will.

About 12 years ago, it was found that lanthanide ions and their complexes are highly active for RNA hydrolysis.[5,6] Especially, Tm(III), Yb(III), and Lu(III) completely hydrolyze RNA under physiological conditions within an hour. Various organic and inorganic scissors for RNA scission were also reported.[7–10] Furthermore, artificial enzymes for sequence-selective RNA scission were prepared by attaching these molecular scissors to oligonucleotides that are complementary with the substrate RNA (Fig. 1 a).[11–14] In this strategy, the scissors are placed near the target phosphodiester linkage so that this link-

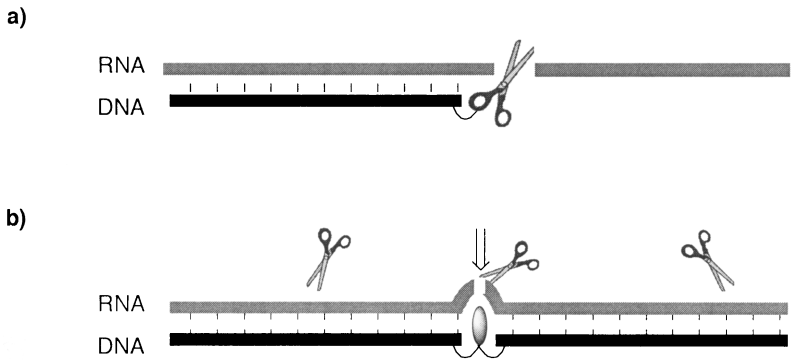



Fig. 1. Two strategies for site-selective RNA scission: (a) conjugation of molecular scissors to oligonucleotides and (b) site-selective activation of RNA by non-covalent interactions. The activated site in (b) is designated by the arrow.

age is selectively cleaved due to a favorable activation-entropy term. Peptide nucleic acids, DNA-binding proteins, and other organic or inorganic molecules are also available as the sequence-recognizing moieties.[15] However, these first-generation artificial enzymes often suffer from a drawback that the catalytic activities of molecular scissors are greatly diminished after their attachment to sequence-recognizing moieties. Still more advanced molecular design is desirable.[16]

Here we present an entirely new strategy for site-selective RNA scission. These second-generation artificial enzymes take advantage of selective activation of the target phosphodiester linkage in the RNA (Fig. 1 b). By using appropriate activators, the conformations of the RNA at the target site are perturbed for efficient scission, and/or steric strain is provided to the target phosphodiester linkage. When some catalysts for RNA hydrolysis are added to these systems, the target phosphodiester linkage is hydrolyzed preferentially over the others, due to a favorable activation-enthalpy term. Covalent attachment of the molecular scissors to any sequence-recognizing moiety is unnecessary, and accordingly the catalytic activity of molecular scissors is never damaged. Such versatile molecular scissors can be employed here.

### 1. Molecular Design of Novel RNA Cutters

The second-generation artificial ribonucleases reported here are composed of an RNA-activator (chemically modified oligonucleotide(s)) and a catalyst for RNA scission. The type-I RNA-activator is an oligonucleotide which has an acridine in the middle (see Fig. 2 a). On the other hand, the type-II activator is the combination of an oligonucleotide bearing an acridine and an unmodified oligonucleotide (Fig. 2 b).

When the substrate RNA forms a hetero-duplex with the oligonucleotide(s) in the RNA-activator, most of the ribonucleotides in the RNA form Watson–Crick base pairs with the counterpart nucleotides. However, the ribonucleotide, which is located in front of the acridine, remains unpaired. As the result, the phosphodiester linkages adjacent to this unpaired ribonucleotide are selectively activated, and are preferentially hydrolyzed by molecular scissors (lanthanide and non-lanthanide ions) over the other linkages. This site-selective acti-

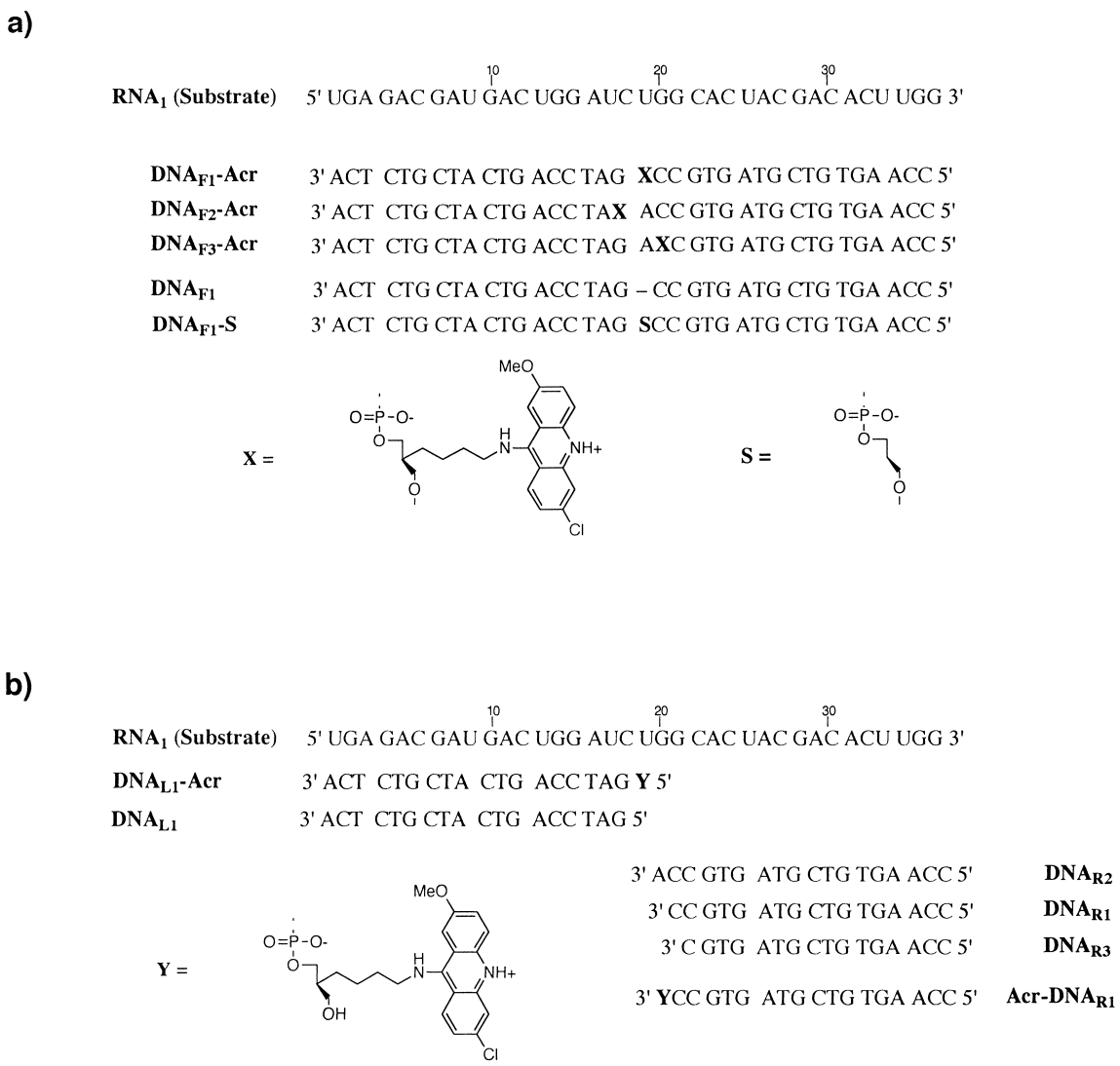



Fig. 2. Structures of the type-I RNA-activators (a) and the type-II RNA-activators (b).

vation is so enormous that the scission is prompt even with intrinsically poor molecular scissors.

## 2. Site-Selective RNA Scission by Lanthanide Ions[17–19]

**2.1 Type-I RNA-Activators.** As shown in Fig. 2 a, $DNA_{F1}$-Acr has an acridine moiety in its internal position, and is complementary with most of the substrate $RNA_1$. In the $RNA_1/DNA_{F1}$-Acr hetero-duplex, only the ribonucleotide U19 (in front of the acridine) is kept free from Watson–Crick base-pairing. When this system is treated with Lu(III) chloride (one of the most active catalysts for RNA hydrolysis),[5] the $RNA_1$ is site-selectively and efficiently hydrolyzed (lane 9 in Fig. 3). The selective scission-sites are the 5′-side of U-19 (major-site) and its 3′-side (minor-site), as schematically depicted in Fig. 4 a. At pH 7 and 37 °C, the scission is also efficient, and more than half of the $RNA_1$ is cleaved within 6 h. The site-selectivity is satisfactorily high throughout the whole process. In place of Lu(III), other lanthanide(III) ions can be also used for this site-selective scission (lanes 3 and 6). The activity at pH 8 is as follows: Ho(III), Dy(III), Tb(III), Gd(III) > Lu(III), Yb(III), Tm(III), Er(III) > Sm(III), Eu(III) > La(III) > Ce(III) > Pr(III) > Nd(III).

The scission is marginal when a trimethylene spacer is inserted to $DNA_{F1}$ in front of U19 ($DNA_{F1}$-S: lanes 2, 5, and 8). Such results substantiate the essential role of the acridine residue for the present site-selective scission. What is crucial is not the presence of unpaired ribonucleoside, but the interactions between the acridine and the RNA.

**2.2 Type-II RNA-Activators.** Site-selective scission is also successful, when the type-I activator is divided into an oligonucleotide bearing an acridine and another unmodified oligonucleotide (the type-II RNA-activator). For example, $DNA_{L1}$-Acr is complementary with the 5′-side portion of $RNA_1$ and bears an acridine at the 5′-end, while $DNA_{R1}$ is complementary with the 3′-side portion of $RNA_1$ (see Fig. 2 b). Accordingly, only U-19 of $RNA_1$ is unpaired. On the addition of Lu(III), the $RNA_1$ is selectively and efficiently hydrolyzed at the phosphodiester linkage in the 5′-side of U-19 (lane 3 in Fig. 5; see also Fig. 4 b). Another weak scission occurs at its 3′-side. These scission sites are exactly identical with those using $DNA_{F1}$-Acr as the type-I activator.

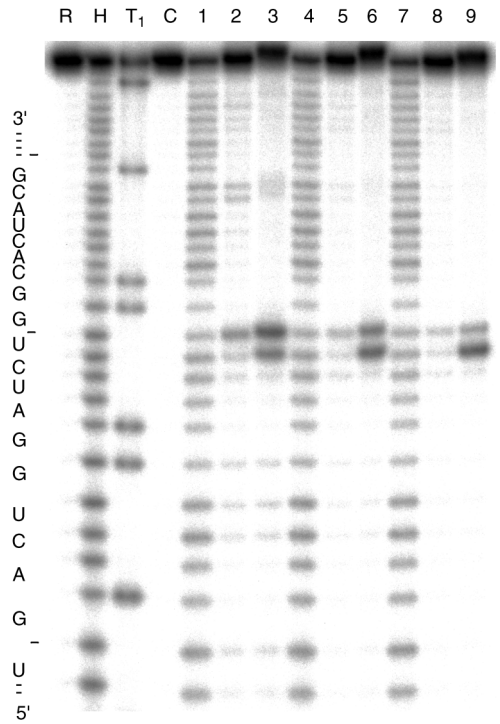



Fig. 3. Site-selective RNA scission by combinations of type-I activator and lanthanide(III) ion. Lane 1, La(III) only; lane 2, $DNA_{F1}$-S/La(III); lane 3, $DNA_{F1}$-Acr/La(III); lane 4, Eu(III) only; lane 5, $DNA_{F1}$-S/Eu(III); lane 6, $DNA_{F1}$-Acr/Eu(III); lane 7, Lu(III) only; lane 8, $DNA_{F1}$-S/Lu(III); lane 9, $DNA_{F1}$-Acr/Lu(III). At pH 8.0 and 37 °C for 2 h; $[RNA_1]_0$ = 1, $[DNA_{F1}\text{-S}]_0$ = $[DNA_{F1}\text{-Acr}]_0$ = 10, and $[LnCl_3]_0$ = 100 μM; $[NaCl]_0$ = 200 mM. R, $RNA_1$ only; H, alkaline hydrolysis; $T_1$, RNase $T_1$ digestion; C, control reaction in buffer solution. M = mol $dm^{-3}$.

The site-selective RNA scission is also successful, when an unmodified oligonucleotide $DNA_{L1}$ is combined with a modified oligonucleotide Acr-$DNA_{R1}$ which bears an acridine at the 3′-end (lane 4 in Fig. 5). The scission efficiency is comparable with that provided by the $DNA_{L1}$-Acr/$DNA_{R1}$/Lu(III) system. Thus, an acridine residue can be attached to either of the two

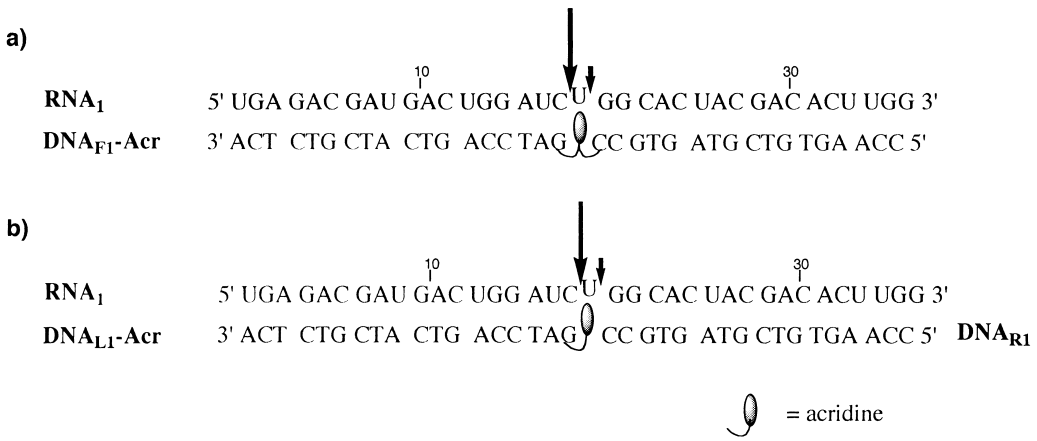



Fig. 4. Site-selective scission by (a) the $DNA_{F1}$-Acr/Lu(III) system and (b) the $DNA_{L1}$-Acr/$DNA_{R1}$/Lu(III) system.

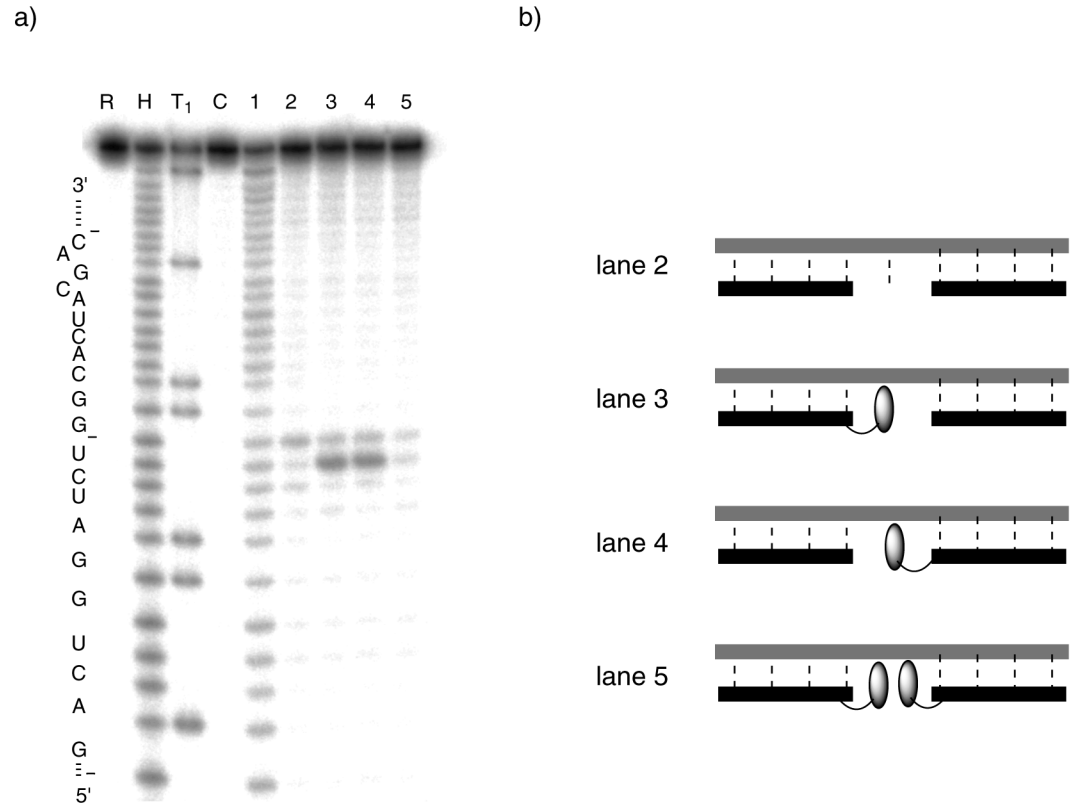



Fig. 5. Site-selective RNA scission by various combinations of type-II activator and Lu(III): (a). Lane 1, Lu(III) only; lane 2, $DNA_{L1}/DNA_{R1}$/Lu(III); lane 3, $DNA_{L1}$-Acr/$DNA_{R1}$/Lu(III); lane 4, $DNA_{L1}$/Acr-$DNA_{R1}$/Lu(III); lane 5, $DNA_{L1}$-Acr/Acr-$DNA_{R1}$/Lu(III). At pH 8.0 and 37 °C for 2 h; $[RNA_1]_0 = 1$, [each of modified or unmodified oligonucleotides]$_0 = 10$, and $[LuCl_3]_0 = 100$ μM; $[NaCl]_0 = 200$ mM. (b) Schematic representations of the combinations used in (a).

oligonucleotides. When both of the oligonucleotides have an acridine residue (the $DNA_{L1}$-Acr/Acr-$DNA_{R1}$ combination), however, the scission is inefficient (lane 5).

### 3. Requirements for the Site-Selective Scission

The acridine group is absolutely indispensable for the present site-selective scission. When unmodified $DNA_{L1}$ is used in place of the $DNA_{L1}$-Acr (together with $DNA_{R1}$), the RNA hydrolysis is far slower (compare lane 2 with lanes 3 and 4 in Fig. 5). The activation of the target site in the RNA is more than 10 fold. Similar results have been obtained for all the RNA-activators investigated. In contrast, the other phosphodiester linkages in the substrate RNA are deactivated on the formation of hetero-duplexes with the "activators". Because of the cooperation of these two factors, the site-selectivity is quite evident.

When only $DNA_{L1}$-Acr or $DNA_{R1}$ is used alone, the RNA is cleaved randomly throughout the region which remains single-stranded without the coverage by the oligonucleotides (lanes 5 and 6 in Fig. 6). No activation by the acridine occurs at either U-19 or the other sites. In order to activate RNA satisfactorily (and achieve the site-selective scission), both of these oligonucleotides are necessary and, in addition, one of them must have an acridine residue at the end. Both the chloro- and methoxy-groups on the acridine are also important (although not essential), since the removal of them decreases the activity by 3–8 fold (Table 1).[20,21] As expected, the Lu(III) ion itself (as well as all the other metal ions used in this paper) has no specific sequence-selectivity (see lanes 1, 4, and 7 in Fig. 3).

In all the non-covalent systems for site-selective scission described above, one nucleotide is removed from the completely complementary oligonucleotide and an acridine is introduced there. The unpaired ribonucleotide in the RNA is the target for the selective scission. With two ribonucleotides unpaired by using the $DNA_{R3}/DNA_{L1}$-Acr combination, the scission is also selective (lane 4 in Fig. 6). When $DNA_{R2}$ (in place of $DNA_{R1}$) is combined with $DNA_{L1}$-Acr, however, the scission is virtually nil (lane 2). Here, all the ribonucleotides in the RNA form Watson–Crick base pairs with the nucleotides. One or two ribonucleotides must be kept unpaired for the present selective reactions.

### 4. No Limitation of the Sequence at the Target Site

When the position of acridine in the DNA/RNA hetero-duplex is moved by using appropriate oligonucleotide(s), the selective-scission site is accordingly altered (Fig. 7). The scission efficiency values are not much different from each other. Thus, the present site-selective scission is successful irrespective of the sequence at the scission-site. These arguments are further confirmed by a systematic study using a series of RNA substrates (data not shown). The site-selective RNA scission by other metal ions is also less dependent on the sequence. This is one of the most important advantages of the present non-covalent systems, since conventional ribozymes usually

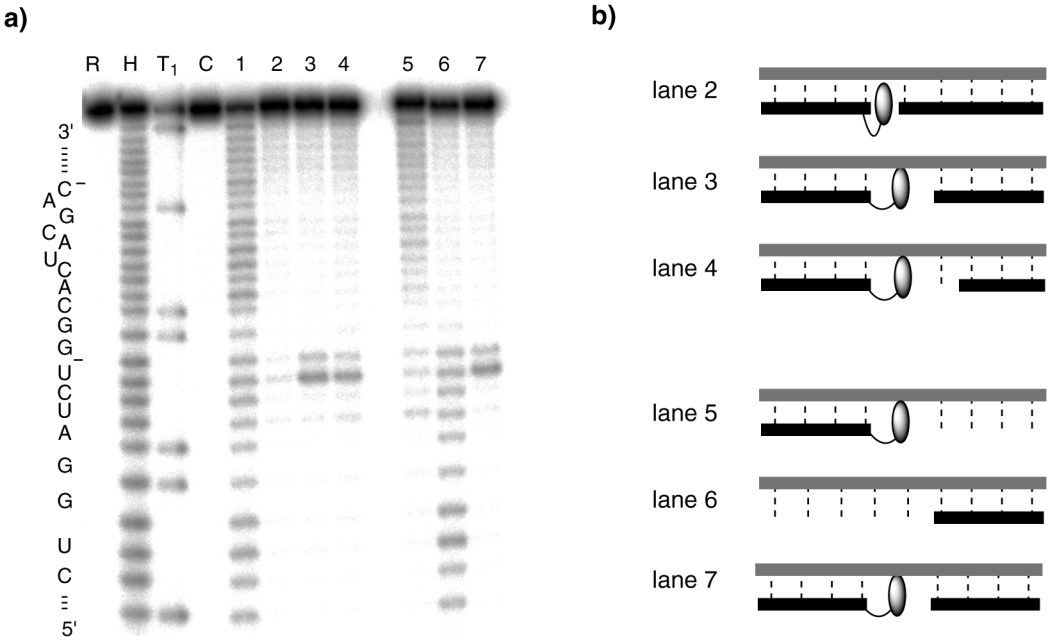
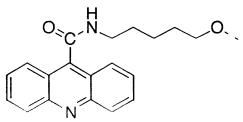
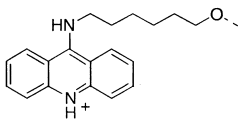
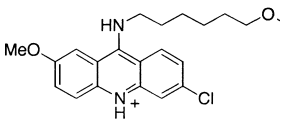



Fig. 6. Effect of the number of unpaired ribonucleotides ($n$) on the site-selective RNA scission by the combinations of type-II activator and Lu(III): (a). Lane 1, Lu(III) only; lane 2, $DNA_{L1}$-Acr/$DNA_{R2}$/Lu(III) ($n = 0$); lane 3, $DNA_{L1}$-Acr/$DNA_{R1}$/Lu(III) ($n = 1$); lane 4, $DNA_{L1}$-Acr/$DNA_{R3}$/Lu(III) ($n = 2$); lane 5, $DNA_{L1}$-Acr/Lu(III); lane 6, $DNA_{R1}$/Lu(III); lane 7, the same as in lane 3. At pH 8.0 and 37 °C for 2 h; $[RNA_1]_0 = 1$, [each of modified or unmodified oligonucleotides]$_0 = 10$ and $[LuCl_3]_0 = 100$ μM; $[NaCl]_0 = 200$ mM. (b) Schematic representations of the combinations used in (a).

Table 1. Activities of the Type-II Activators Bearing Various Acridine Derivatives for Lu(III)-Induced Site-Selective Scission of $RNA_1$[a)]

| X = | none |  |  |  |
|---|---|---|---|---|
| Activity | 1.0 | 1.8 | 5.2 | 14.5 |

a) The X residue in $DNA_{L1}$-Acr is changed to various acridine derivatives and is combined with $DNA_{R1}$. $[DNA_{L1}\text{-Acr}]_0 = [DNA_{R1}]_0 = 10$ μM; [Lu(III)] = 100 μM at pH 8.0 and 37 °C.

require a specific sequence at the target site. For example, hammerhead ribozymes choose NUX site for the scission (N = A, G, C, T and $X$ = A, C, T).[4]

### 5. Site-Selective RNA Scission by Non-Lanthanide Ions

Various non-lanthanide metal ions can be also used as the molecular scissors. Typical ones are Zn(II), Mn(II), Co(II), Ni(II), Cu(II), Cd(II), Mg(II), and Ca(II). These metal ions are intrinsically poor in the activity for RNA hydrolysis. In the present non-covalent systems, however, they show sufficient catalysis.

**5.1 RNA Scission by Zn(II) and Other Transition Metal Ions**[19] When Zn(II) is combined with $DNA_{F1}$-Acr, the $RNA_1$ is selectively hydrolyzed at the 3′- and 5′-sides of U-19 (lane 5 in Fig. 8). These scission-sites are located exactly in front of the acridine in the $RNA_1$/$DNA_{F1}$-Acr hetero-duplex (see Fig. 2 b). The site-selective scission is satisfactorily prompt, and more than half of the RNA is hydrolyzed in 16 h at 37 °C and pH 8.0. It is noteworthy that most of Zn(II) complexes previously reported are poor for RNA hydrolysis (only specially designed dinuclear and trinuclear Zn(II) complexes show the catalysis effect).[9] In the present systems, however, Zn(II) ion is free from the complex formation with strong ligands and satisfactorily retains the activity. Furthermore, the target phosphodiester linkage is activated by the conformational change of RNA. These two factors facilitate reactions which otherwise do not proceed easily. Cu(II), Ni(II), Co(II), and Mn(II) ions are also available as the molecular scissors. Without $DNA_1$-Acr, however, all these metal ions are only marginally active.

The site-selective scission is also successful with the $DNA_{L1}$-Acr/$DNA_{R1}$ combination as the type-II RNA-activator (lane 4). The indispensable role of acridine moiety is clearly evidenced by the following two results. (1) When $DNA_{L1}$ having no acridine is combined with $DNA_{R1}$, the RNA cleavage is virtually nil (lane 2); (2) $DNA_{F1}$-S is inactive (lane 3).

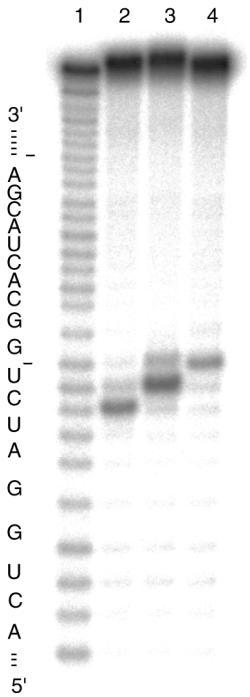



Fig. 7. Effect of the sequence of target site on the site-selective RNA scission. Lane 1, Lu(Ⅲ) only; lane 2, $DNA_{F2}$-Acr/Lu(Ⅲ); lane 3, $DNA_{F1}$-Acr/Lu(Ⅲ); lane 4, $DNA_{F3}$-Acr/Lu(Ⅲ). At pH 8.0 and 37 °C for 2 h; $[RNA_1]_0 = 1$, [modified oligonucleotide]$_0 = 10$, and $[LuCl_3]_0 = 100$ μM; $[NaCl]_0 = 200$ mM.

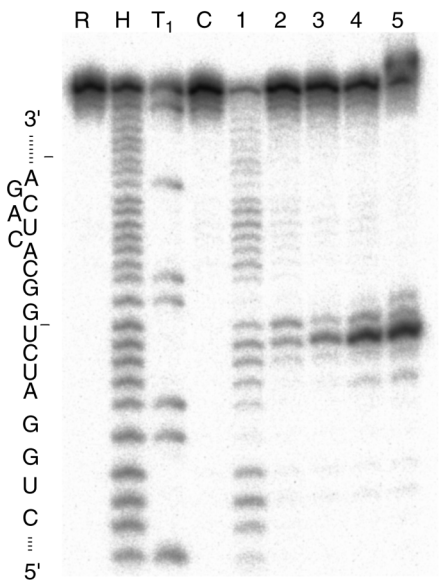



Fig. 8. RNA scission by the combinations of various oligonucleotides and Zn(Ⅱ). Lane 1, Zn(Ⅱ) alone; lane 2, $DNA_{L1}/DNA_{R1}$/Zn(Ⅱ); lane 3, $DNA_{F1}$-S/Zn(Ⅱ); lane 4, $DNA_{L1}$-Acr/$DNA_{R1}$/Zn(Ⅱ); lane 5, $DNA_{F1}$-Acr/Zn(Ⅱ). At pH 8.0 and 37 °C for 16 h; $[RNA]_0 = 1$ and [each of modified or unmodified DNAs]$_0 = 10$ μM; $[Zn(NO_3)_2]_0 = 1$ mM; $[NaCl]_0 = 200$ mM.

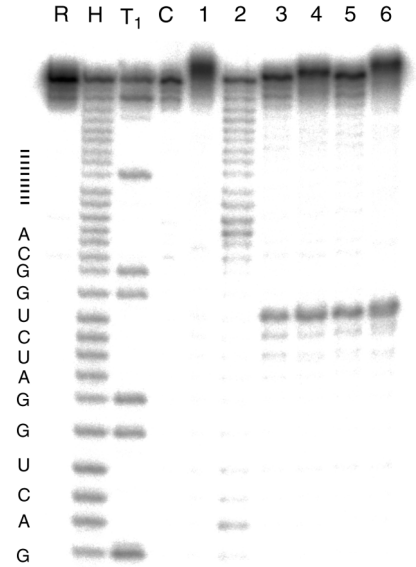



Fig. 9. RNA scission by the combinations of various oligonucleotides and Mg(Ⅱ). Lane 1, $DNA_{F1}$-Acr only; lane 2, Mg(Ⅱ) only; lane 3, $DNA_{L1}/DNA_{R1}$/Mg(Ⅱ); lane 4, $DNA_{F1}$-S/Mg(Ⅱ); lane 5, $DNA_{L1}$-Acr/$DNA_{R1}$/Mg(Ⅱ); lane 6, $DNA_{F1}$-Acr/Mg(Ⅱ). At pH 8.0 and 37 °C for 16 h; $[RNA]_0 = 1$ and [each of modified or unmodified DNAs]$_0 = 10$ μM; $[Mg(Ⅱ)]_0 = 320$ mM; $[NaCl]_0 = 200$ mM.

**5.2 RNA Scission by Mg(Ⅱ) and Ca(Ⅱ)**[22] As depicted in Fig. 9, Mg(Ⅱ) ion is also employable for the site-selective scission. This metal ion is abundant in cells, and is used as a cofactor by many enzymes and ribozymes. With the $DNA_{F1}$-Acr/Mg(Ⅱ) combination, the phosphodiester linkage in the 3′-side of the unpaired U-19 is selectively and efficiently hydrolyzed (lanes 5 and 6). The present selective cleavage is far more efficient than is the random cleavage of single-stranded RNA in lane 2. The RNA is activated on the formation of the RNA/DNA hetero-duplex. The $DNA_{F1}$-Acr/Ca(Ⅱ) combination is also active. However, the $DNA_{L1}/DNA_{R1}$ combination gives only a poorer result (lane 3).

## 6. Mechanism of the Site-Selective Scission

Spectroscopic analysis (fluorescence, UV, and CD) has shown that, in the DNA/RNA hetero-duplexes, the acridine groups are sandwiched between the two Watson–Crick base pairs (see Fig. 10). The acridine is so large in size that it cannot be simultaneously accommodated together with the unpaired RNA-base (U-19 in Fig. 2 a). As the result, the unpaired RNA-base is pushed out from the hetero-duplex, perturbing the conformation of the target site in the RNA.[23] The 2′-OH at the scissile linkage is located near the corresponding phosphorus atom, and its intramolecular nucleophilic attack is greatly promoted. This mechanism is similar to that proposed for the scission by hammerhead ribozymes.[24]

It is noteworthy that RNA is never activated when a bulge structure is formed by using partially complementary oligonucleotide $DNA_{F1}$ having no acridine group. Apparently, this simple bulge-strategy is useless for the site-selective RNA scission. The strain energy is widely spread over many phosphodiester linkages and thus the activation of target linkage(s)

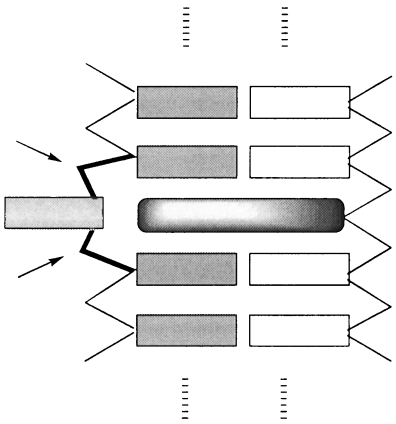


Fig. 10. Proposed mechanism of site-selective scission by the present non-covalent RNA cutters. The acridine is shown by a rounded rectangle, and the activated phosphodiesters are by arrows.

is marginal. In the present non-covalent systems, however, the acridine is firmly placed between the Watson–Crick base pairs in the hetero-duplex, and thus the conformational change by the acridine is concentrated onto the target phosphodiester linkage.

## 7. Conclusion

Novel sequence-selective RNA cutters are synthesized by combining modified oligonucleotide(s) with catalysts for RNA hydrolysis. The target phosphodiester linkage is activated and hydrolyzed preferentially over the other linkages. The scission requires no specific sequence, and thus any target site can be selectively hydrolyzed with a desired specificity. This is a great advantage of these systems over conventional ribozymes. Other advantages are (1) sterically hindered RNA can be effectively hydrolyzed and (2) various chemical and physiological functions can be easily provided by chemical modification. As the molecular scissors, Zn(II) and Mg(II) ions which are rather abundant in cells are also available. Accordingly, the way to versatile applications for molecular biology, biotechnology, and therapy is being paved. Moreover, non-covalent and site-selective activation of the target phosphodiester linkage should be useful to develop new RNA chemistry. Further improvement of the activation efficiency is desirable.

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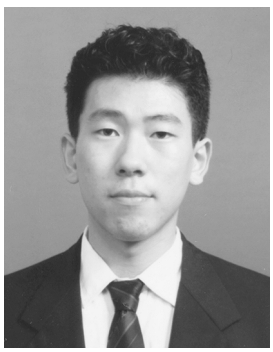

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