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Selective Activation of Two Sites in RNA by Acridine-Bearing Oligonucleotides for Clipping of Designated RNA Fragments

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Abstract: Artificial enzymes for selective scission of RNA at two designated sites, which are valuable for advanced RNA science, have been prepared by combining lanthanide(III) ion with an oligonucleotide bearing two acridine groups. When these modified oligonucleotides form heteroduplexes with substrate RNA, the two phosphodiester linkages in front of the acridines are selectively activated and preferentially hydrolyzed by lanthanide ion. This two-site RNA scission does not require any specific RNA sequence at the scission sites, and the length of clipped RNA fragment is easily and precisely controllable by changing the distance between two acridine groups in the modified oligonucleotide. The two-site scission is also successful even when the substrate RNA has higher-order structures. By using these two-site RNA cutters, RNA fragments of predetermined length were obtained from long RNA substrates and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Single nucleotide polymorphisms in homozygous and heterozygous samples were accurately and easily detected in terms of the difference in mass number. Multiplex analyses of in vitro transcripts from human genome were also successful.

Introduction

In this decade, significant attention has been focused onto nonenzymatic hydrolysis of RNA, and various molecular scissors (e.g., metal ions, oligoamines, and others) were reported.[1] By attaching them to sequence-recognizing oligonucleotides or their equivalents, artificial enzymes for site-selective scission of RNA were prepared.[2,3] Ribozymes also cut RNA at predetermined one site.[4] However, there have been few methods which can freely cut RNA substrate at closely located two designated sites and clip a fragment of desired length and sequence.[5] These methods, if available, should be quite useful for further developments of RNA science. For example, RNA fragments, which involve modified nucleobases (e.g., isopentenyladenine and wybutosine) or take complicated tertiary structures, are easily obtainable from naturally occurring RNA and can be investigated by various analytical and/or biological means. Preparation of chimera of two modules from two sources should be also greatly facilitated. Another promising application of these methods is genotyping of single nucleotide polymorphisms (SNPs) in human genomes.[6,7] By analyzing appropriate RNA fragments by mass spectroscopy, we can obtain definite conclusion on whether a certain nucleotide in the target gene is altered to another one.[8] This information is important for the prediction of hereditary diseases, design of tailor-made medicines, and many other biomedical applications where SNPs are taking important roles.[9]

For efficient clipping of desired RNA fragments from substrate RNA, the following factors are necessary: (1) the scission must selectively take place at the two designated sites, (2) the scission must be prompt irrespective of the RNA sequence, (3) the distance between the two scission sites must

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be easily controllable, and (4) the RNA fragments, prepared by the two-site scission, must be sufficiently protected from subsequent digestion. As shown here, all these requirements are successfully fulfilled by applying our recent finding of non-covalent strategy for site-selective RNA scission.[10] This strategy is based on site-selective activation of RNA by an oligonucleotide bearing an acridine, which is induced upon the duplex formation between them. The phosphodiester linkage of RNA in front of the acridine is selectively activated and hydrolyzed by lanthanide ions or some transition-metal ions such as Zn(II), even when these catalysts are not fixed to any sequence-recognizing moiety. This article reports that the two target sites in substrate RNA are efficiently activated by oligonucleotides bearing two acridines (two-site activators) and are selectively hydrolyzed by lanthanide ions. These activations at the two sites occur almost independently from each other. The clipping efficiency is hardly dependent on either the kind of nucleobases in front of the acridines or the sequence near the scission sites, and also, RNA having higher-order structures can be the substrates for the two-site scission. Thus, any predetermined fragment of desired length can be clipped from various RNA substrates in high yields. Furthermore, the present two-site RNA scissions are used for SNP genotyping in which the fragments from genomic samples are analyzed by MALDI-TOF MS.

Results and Discussion

Selective Scission of RNA at Two Designated Sites. Figure 1 shows the results of polyacrylamide gel electrophoresis (PAGE) for the scission of a 40-mer RNA substrate (R_1G) at 37 °C by Lu(III) ion (the sequences of R_1G and the activators are presented in (A)). This model substrate has the antisense sequence of a part of the exon of the human apolipoprotein E gene (*apoE*) and involves a potential SNP site at G290. The numbering is based on that in the sense-DNA strand and thus increases from the 3′-end of RNA to the 5′-end. The melting temperatures (T_m) of the duplexes of R_1G with all the activators used (D_1X_2, D_1X_{1a}, or D_1X_{1b}) are higher than 80 °C. To follow the two-site scission precisely, R_1G was labeled both at the 5′-end (with fluorescein: FAM) and at the 3′-end (with tetramethylrhodamine: TMR). In lane 4 in Figure 1B, the modified oligonucleotide D_1X_2 was used as activator, and the scission was detected by the fluorescence from the FAM (the excitation at 473 nm). This activator is complementary with R_1G in the most part, but bears two acridines in front of U298 and U285. The scission of R_1G selectively occurred both at U298 (the solid arrow) and at U285 (the dotted arrow). Thus, the phosphodiester linkages in front of these two acridines were selectively activated and hydrolyzed by the Lu(III) ion. In Figure 1C, the same reaction was analyzed by the fluorescence from the TMR at the 3′-end of R_1G (the excitation at 532 nm). The selective scission of R_1G at the two designated sites was further confirmed. As expected, when either D_1X_{1a} or D_1X_{1b} was used in place of D_1X_2, only one phosphodiester linkage at the corresponding site was hydrolyzed (lanes 2 and 3 in Figure 1, parts B and C). These modified oligonucleotides have an acridine in front of one of the two target sites but take the complementary nucleotide A in front of the other site.

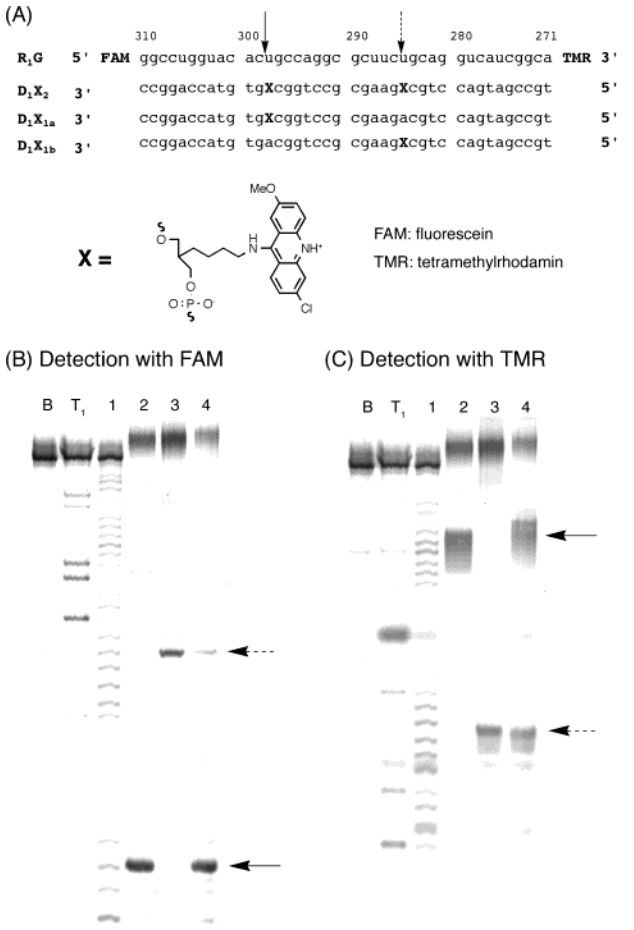

Figure 1. PAGE patterns for the two-site scission of R_1G by the combination of oligonucleotide bearing two acridines and a Lu(III) ion. For both of the patterns in (B) and (C): lane 1, Lu(III) only; lane 2, D_1X_{1a}/Lu(III); lane 3, D_1X_{1b}/Lu(III); lane 4, D_1X_2/Lu(III); B, buffer reaction; T_1, RNase T_1 digestion. The PAGE in (B) was monitored by the fluorescence from the FAM at the 5′-end of R_1G, whereas the PAGE in (C) was analyzed by using the TMR at the 3′-end. The structures of RNA substrate and the activators are presented in (A). The target scission sites are designated by the arrows. Reaction conditions: pH 7.5 and 37 °C for 3 h; $[R_1G]_0 = 2.0$, [the activator] = 10.0, and [Lu(III)] = 150 μM; [NaCl] = 200 mM. Mobility of R_1G and the products in the gels (especially in (C)) were slightly suppressed by coexisting modified oligonucleotides. Because of the high self-complementarity, linkages between G297 and C291 are not sufficiently cleaved by free Lu(III) nor RNase T_1 as in lanes 1 and T_1. Accordingly, all the products were further characterized by MALDI-TOF MS (see text for details).

The products of the RNA hydrolysis by the D_1X_2/Lu(III) combination were directly analyzed by MALDI-TOF MS.[11] As shown in Figure 2B, three peaks were clearly observed. The signal at m/z = 4168.8 is assignable to the 13-mer fragment

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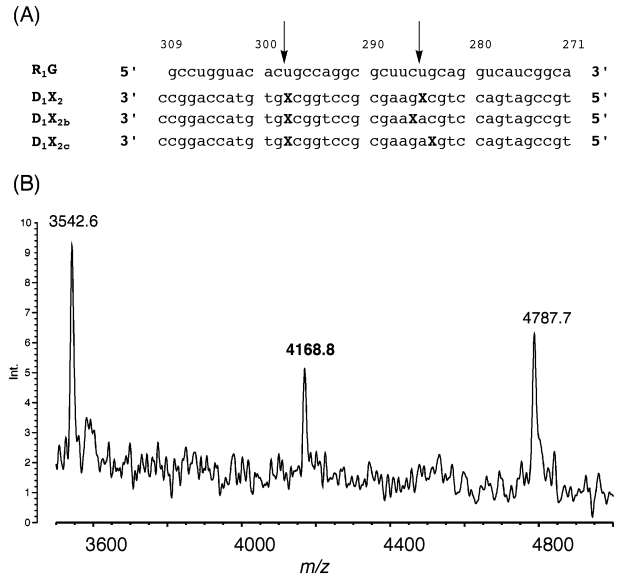

Figure 2. MALDI-TOF MS spectrum for the product of site-selective scission of R_1G by Lu(III) in the presence of D_1X_2 (the sequences are presented in (A)).[11] The signal at 4168.8 corresponds to the 13-mer fragment (U298−C286). The signals at 3542.6 and 4787.7 are for the 5′-side fragment (G309−C299) and the 3′-side fragment (U285−A271), respectively, and were used as the internal standards for precise determination of mass number of the 13-mer fragment. The mass analysis was performed in negative ion mode. Reaction conditions: pH 7.5 and 37 °C for 3 h; $[R_1G]_0 = 2.0$, [the activator] = 10.0, and [Lu(III)] = 150 μM; [NaCl] = 200 mM. The MS spectrum of the product in the presence of D_1X_{2b} is presented in the Supporting Information.

ranging from U298 to C286, which has both 5′-OH and 3′ (or 2′)-monophosphate ends (the theoretical value for $[M - H]^-$ is 4169.5). The other two signals (m/z = 3542.6 and 4787.7) are for the 5′-side portion of the substrate RNA (G309-C299) and its 3′-side portion (U285-A271), respectively, which were used as the internal standards for precise determination of the mass number of the 13-mer fragment. It has been concretely substantiated that the phosphodiester linkages in the 5′-sides of both U298 and U285 were efficiently activated by the confronting acridines and were hydrolyzed by the Lu(III) ion. Moreover, the 13-mer RNA fragment formed by this primary scission at these two sites was successfully protected by the modified oligonucleotide D_1X_2 from the subsequent digestion. Otherwise, this RNA fragment, even if it is formed, should be promptly digested by the Lu(III) ion in the solutions. The melting temperature of the heteroduplex between this 13-mer fragment and the corresponding portion in D_1X_2 is consistently 62 °C, which is significantly higher than the reaction temperature (37 °C). Thus, the duplex formation is almost completed under the reaction conditions, and the 13-mer fragment is sufficiently stable in this duplex.[12] Other lanthanide(III) ions (e.g., La(III) and Eu(III)) could be also successfully used as the catalyst, although they were slightly less active than Lu(III) (data not shown).

Efficiency of the Two-Site Selective RNA Scission. The amount of the 13-mer RNA fragment produced by the present

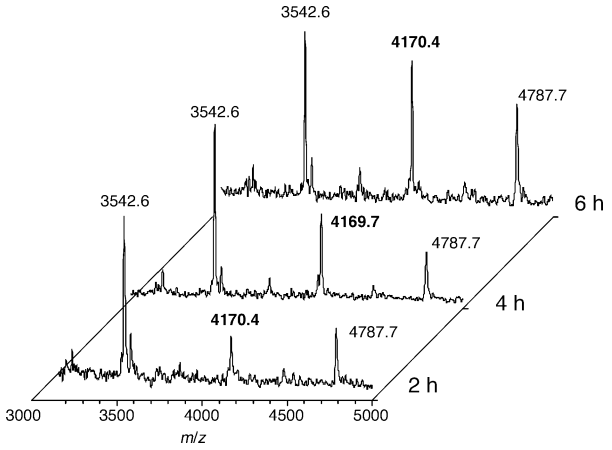

Figure 3. Time-course for the formation of the 13-mer fragment (U298−C286) from R_1G by the D_1X_2/Lu(III) combination. The products by the reaction in Figure 2 were analyzed by MALDI-TOF MS at different reaction times. Minor signals (e.g., signals at 3236.6, 3869.8, and 4479.8) are from non-selective cleavages.

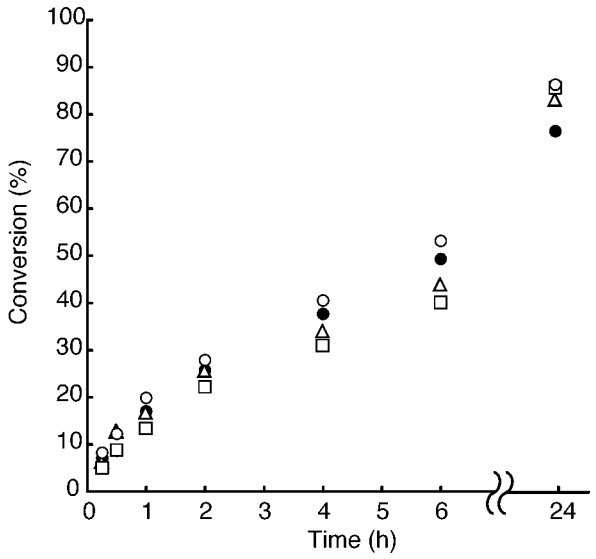

Figure 4. Time dependencies of conversion for the scission of R_1G at U298 by Lu(III) in the presence of D_1X_2 (○), D_1X_{1a} (●), D_1X_{2b} (△), or D_1X_{2c} (□). The scission was detected by using the fluorescence from the FAM at the 5′-end of R_1G. Reaction conditions: pH 7.5 and 25 °C; $[R_1G]_0$ = 2.0, [the activator] = 10.0, and [Lu(III)] = 150 μM; [NaCl] = 200 mM.

two-site scission monotonically increased with increasing reaction time (Figure 3). According to the PAGE analysis using the fluorescence from the FAM, the yield of the 5′-side fragment after 6 h at 37 °C is 78 mol %. In MS analysis, the ratio of the intensity of the signal for this fragment (m/z = 3542.6) to that for the desired 13-mer fragment (m/z = 4170.4) is 1.2. Thus, the yield of the desired fragment, with respect to the substrate RNA, is around 60 mol % at 6 h and is satisfactorily high for various applications. As expected, the RNA scission was faster at higher temperatures. For example, the reaction at 50 °C was about 3 times as fast as that at 37 °C. The selectivity of two-site scission was kept high.

The open circles in Figure 4 show the time dependence of conversion for the hydrolysis of R_1G at the phosphodiester linkage in the 5′-side of U298 by the D_1X_2/Lu(III) combination (the main band in the bottom of lane 4 in Figure 1B). At 24 h, sufficiently high conversion (80 mol %) is accomplished. Note that this kinetic analysis is achieved at 25 °C and that the

(11) In this analysis, the 5′-terminal G was removed from R_1G simply to facilitate the analysis. The labeling by either FAM or TMR was not employed here. The products were desalted as described in the Experimental Section and directly subjected to the MALDI-TOF MS.

(12) The RNA in DNA/RNA duplexes is considerably stable against the digestion by lanthanide ions: Kolasa, K. A.; Morrow, J. R.; Sharma, A. P. *Inorg. Chem.* **1993**, *32*, 3983−3984.

reaction time can be shortened at higher temperatures. Significantly, the rate of hydrolysis at U298 by D_1X_2 is almost identical with that by the one-site activator D_1X_{1a}, which bears only one acridine in front of U298 (compare the open circles with the closed ones). In the two-site activator D_1X_2, the activations by the two acridines occur in a close proximity in the helical heteroduplex, but proceed almost independently from each other. Other two-site activators (D_1X_{2b} and D_1X_{2c}), which have the first acridine in front of U298 but the second one at the position other than U285 (see Figure 2A), are consistently almost as active as D_1X_2 (the open triangles and squares vs the open circles).

Effects of the Length and the Sequence between the Two Acridines on the Two-Site Selective RNA Scission. In Figure 1, there exist 12 nucleotides between two acridines in the two-site activator D_1X_2, and the 13-mer fragment is selectively clipped from the target position. The RNA fragment is longer by one nucleotide than the DNA portion between the acridines in the activator, since the scission occurs at the phosphodiester linkages in the 5′-sides of both of the target nucleotides (note that these acridines are incorporated to the two-site activator in exchange for the corresponding complementary nucleotide). A 12-mer RNA fragment was consistently prepared in high yield, when the number of nucleotides between the acridines was decreased to 11 (see Supporting Information). Similarly, 9-, 10-, 11-, 14-, and 15-mer RNA fragments were selectively prepared, respectively, by use of the two-site activators which involved 8, 9, 10, 13, and 14 nucleotides between two acridines (the formation of 10-mer fragments is presented in Figure 6). In all the cases, the site for the selective scission by Lu(III) is the 5′-side of each of the nucleotides which confront the two acridines. The efficiencies of two-site scission in these experiments were similar, even though the target sequences were entirely different from each other. The nucleobase in front of the acridine could be any of A, G, C, and U, and similar scission rates were obtained for all of them.[13] Accordingly, RNA fragments of desired length are selectively obtainable by the present method, irrespective of the sequence around the two scission sites. The present site-selective activation by the activators is not associated with direct hydrogen-bonding interactions of the acridines with the nucleobases.[10]

Precise Clipping in the Presence of Mismatch between the RNA Substrate and the Activator. In all the examples hitherto described, the whole part of two-site activator (except for the acridines) was completely complementary to the target RNA fragment. Under these conditions, the fragments, produced by the primary scission at the two sites, were successfully protected from the subsequent digestion by forming heteroduplexes with the activators. Thus, their yields were satisfactorily high. When there exists a mismatch between the target RNA fragment and the two-site activator, however, the RNA fragment is only poorly protected by the activator and easily digested by the lanthanide ions in the reaction mixtures. For example, when G290 in R_1G was replaced by adenosine and this RNA (R_1A) was treated with Lu(III) in the presence of D_1X_2, the yield of the target 13-mer fragment was only marginal. This critical effect of a mismatch is unfavorable for some of practical applications, since the detailed sequences of RNA samples are scarcely available prior to our analysis. In SNP genotyping, of course, genotype of subject is unknown.

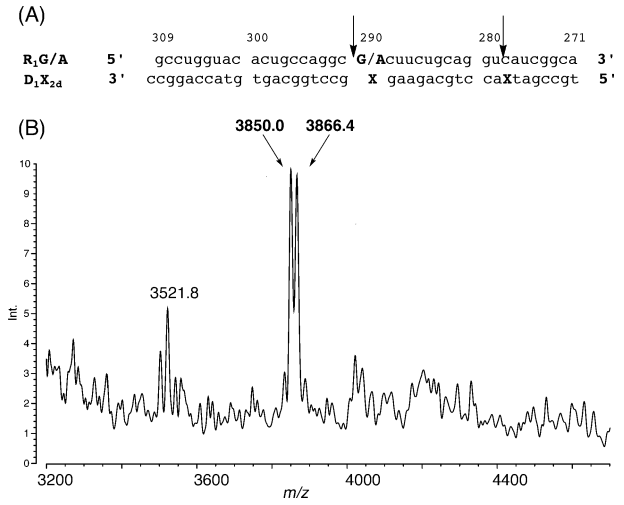

Figure 5. MALDI-TOF MS spectrum for the products of two-site selective scission of 1:1 mixture of R_1G and R_1A in the presence of D_1X_{2d} (the sequences are presented in (A)). The signal at 3850.0 is for the A290−U279 fragment from R_1A, and the signal at 3866.4 is the G290−U279 fragment from R_1G. The 3′-side fragment (C278−U271) and the 5′-side fragment (G309−C291) were detected at 2506.4 and 6127.7, respectively (data not shown). The minor signal at 3521.8 is probably assignable to the fragment produced by the removal of the 5′-terminal nucleotide of the desired fragment in the secondary scission. Reaction conditions: pH 7.5 and 25 °C for 24 h; $[\text{total RNA}]_0 = 4.0$, [the activator] = 10.0, and [Lu(III)] = 150 μM; [NaCl] = 200 mM.

This problem can be successfully solved by taking the mismatching site to one of the two target sites.[14] As demonstrated above, the present activators efficiently activate the target sites irrespective of the kind of the nucleotide in front of the acridine, and the selective hydrolysis always occurs at the phosphodiester linkage in the 5′-side of this nucleotide. Accordingly, sufficient amounts of RNA fragments were obtained from the mixture of R_1G and R_1A, simply by using D_1X_{2d} as the activator and placing one of the two acridines in front of the G of R_1G (or the A of R_1A). As depicted in Figure 5B, two MS signals were clearly detected at $m/z = 3850.0$ and 3866.4, which are assignable to the 12-mer fragment from R_1A and R_1G, respectively (the theoretical values are 3849.6 and 3865.5). It is noteworthy that these fragments contain the nucleotide at the mismatching site (G or A in the above case). Because of this feature, various types of alteration of nucleobases in substrate RNA (alterations of purine ↔ purine, pyrimidine ↔ pyrimidine, and purine ↔ pyrimidine) are easily analyzable.[15]

Application of the Present Two-Site Scission to Genotyping of SNPs by Mass Spectroscopy. The RNA substrates R_2A and R_2G are from the antisense strand of the exon of the apolipoprotein E (*apoE*) gene, and the alteration of the nucleotide from A (the isoform *apoE* 3) to G (*apoE* 4) is related to Alzheimer's disease.[9] Figure 6 is a typical example of SNP genotyping which utilizes the present RNA clipping method.

(13) The site-selective scission was successful even when the target nucleotide in RNA was abasic (see Supporting Information).

(14) Another solution to this problem is to use nonnatural nucleotide dP, which forms stable base pairs both with G and with A through its tautomerism (see Kuzuya, A.; Mizoguchi, R.; Morisawa, F.; Komiyama, M. *Chem. Commun.* **2003**, 770−771).

(15) To discriminate C/U(T) alteration in genomes, the most appropriate way is to use the complementary strand (sense or antisense strand) as the template for in vitro transcription. Then this alteration can be analyzed in terms of G/A alteration, which gives rise to a mass difference of 16 (the difference between C and U is ca. 1).

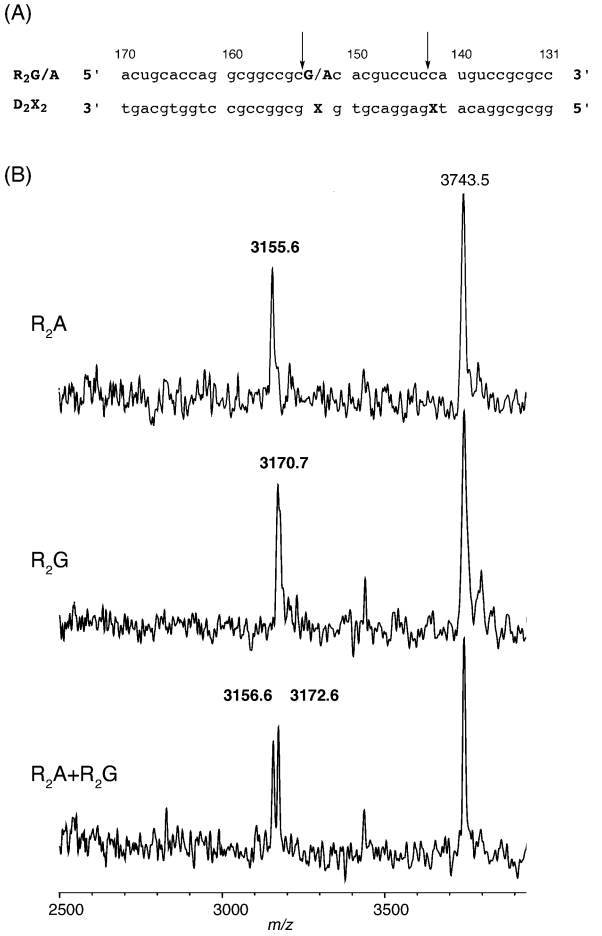

Figure 6. Genotyping of SNPs by using the present two-site RNA scission. In the presence of D_2X_2, the substrate RNA (R_2A, R_2G, or 1:1 mixture of them) was treated with Lu(III), and the products were analyzed by MALDI-TOF MS. The signal at 3155.6 (or 3156.6) is for the 10-mer fragment (A152−C143) from R_2A, and the signal at 3170.7 (or 3172.6) is for the fragment from R_2G. The 3′-side fragment (C142−C131) is observable at m/z = 3743.5. Reaction conditions: pH 7.5 and 25 °C for 24 h; [total RNA]$_0$ = 4.0, [the activator] = 10.0, and [Lu(III)] = 150 μM; [NaCl] = 200 mM.

In the presence of D_2X_2, the substrate RNA [R_2A, R_2G (homozygous samples) or 1:1 mixture of these two RNA (heterozygous sample)] was treated with Lu(III) and analyzed by MALDI-TOF MS. One of the two acridines in D_2X_2 is placed in front of the potential SNP site 152 (Figure 6A). When R_2A was used as the substrate, a sharp signal was detected at m/z = 3155.6, which corresponds to the desired 10-mer fragment (Figure 6B: the theoretical m/z = 3157.5). Similar treatment of R_2G gave the MS signal at m/z = 3170.7 (the theoretical m/z = 3173.4). When the 1:1 mixture of these two RNA was treated as above and analyzed by MALDI-TOF MS, two signals of similar intensities were detected at m/z = 3156.6 and 3172.6 (see the bottom spectrum in Figure 6B). They are assignable to the 10-mer fragment from R_2A and the one from R_2G, respectively. Apparently, both of the RNA substrates were successfully hydrolyzed at the two designated sites, and the corresponding 10-mer fragments were formed in sufficient amounts. On the other hand, a 1:1 mixture of R_1A and R_1G (heterozygous SNP system from the *apoE* gene involving another SNP site 290) also provided two well-separated signals, as already shown in Figure 5. In both figures, the difference in mass number between the two signals is consistent with the

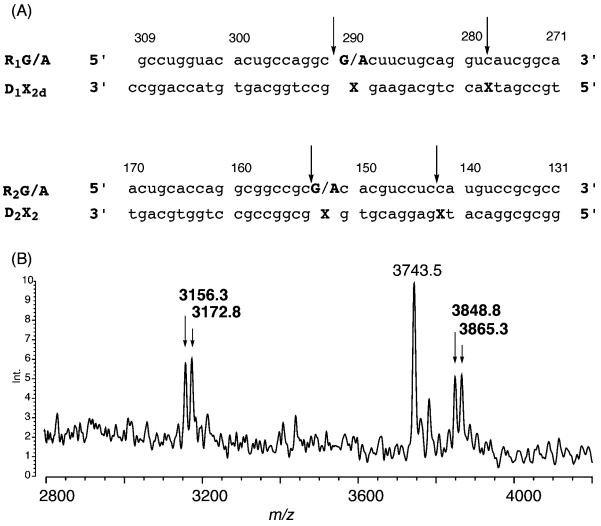

Figure 7. Multiplex genotyping by using the present two-site RNA scission. In the presence of both D_1X_{2d} and D_2X_2, an equimolar mixture of R_1G, R_1A, R_2G, and R_2A (their sequences are presented in (A)) was treated with Lu(III) at pH 7.5, 25 °C for 24 h, and the products were analyzed by MALDI-TOF MS. The signal at 3865.3 is for the 12-mer fragment (G290−U279) from R_1G and that at 3848.8 is from R_1A, while the signal at 3172.8 is for the 10-mer fragment (G152−C143) from R_2G and that at 3156.3 is from R_2A. Two SNP sites in the *apoE* gene are simultaneously analyzed. The 3′-side fragment (C142−C131) is observable at m/z = 3743.5. Reaction conditions: [each RNA]$_0$ = 2.0, [each activator] = 10.0, and [Lu(III)] = 150 μM; [NaCl] = 200 mM.

value (16.0) between A and G. The SNP at the position 290 (A in *apoE* 2 and G in *apoE* 3) is associated with hyperlipemia.[9]

The present genotyping method was also applicable to multiplex assays, where two or more different SNP sites are simultaneously analyzed. In the presence of both D_1X_{2d} (in Figure 5A) and D_2X_2 (in Figure 6A), an equimolar mixture of R_1G, R_1A, R_2G, and R_2A, all of which are from two different parts of the *apoE* gene, was treated with Lu(III). To facilitate the discrimination in MALDI-TOF MS, the lengths of the desired fragments were intentionally made different by using two-site activators which involve different numbers (9 and 11) of nucleotides between the two acridines. As presented in Figure 7, all the expected four fragments [the 12-mer fragments from R_1G (m/z = 3865.3) and R_1A (3848.8), as well as the 10-mer fragments from R_2G (3172.8) and R_2A (3156.3)] were explicitly detected. The theoretical m/z values are 3865.5, 3849.6, 3173.4, and 3157.5, respectively. These results definitely conclude that this subject is heterozygote of *apoE* 2 and *apoE* 4, as expected.

Clipping of Two or More Fragments from Large RNA Having Higher-Order Structure and SNP Genotyping Therein. With the present method, even naturally occurring RNA, which takes notable higher-order structures, can be successfully cut at the two designated sites. For example, desired small fragments were successfully obtained from the exon RNA (179-mer) of the human *apoE* gene, which was prepared from genome by in vitro transcription. This RNA is highly self-complementary and forms a long hairpin loop structure with several internal loops and bulges (the most stable secondary structure under the reaction conditions, calculated by *mfold*,[16] is presented in Figure 8B; its magnified version is available in the Supporting Information). In the isoform employed for the genotyping study (*apoE* 3), the nucleotide at one of the two

(16) Zuker, M. *Nucleic Acids Res.* **2003**, *31*, 3406−3415.

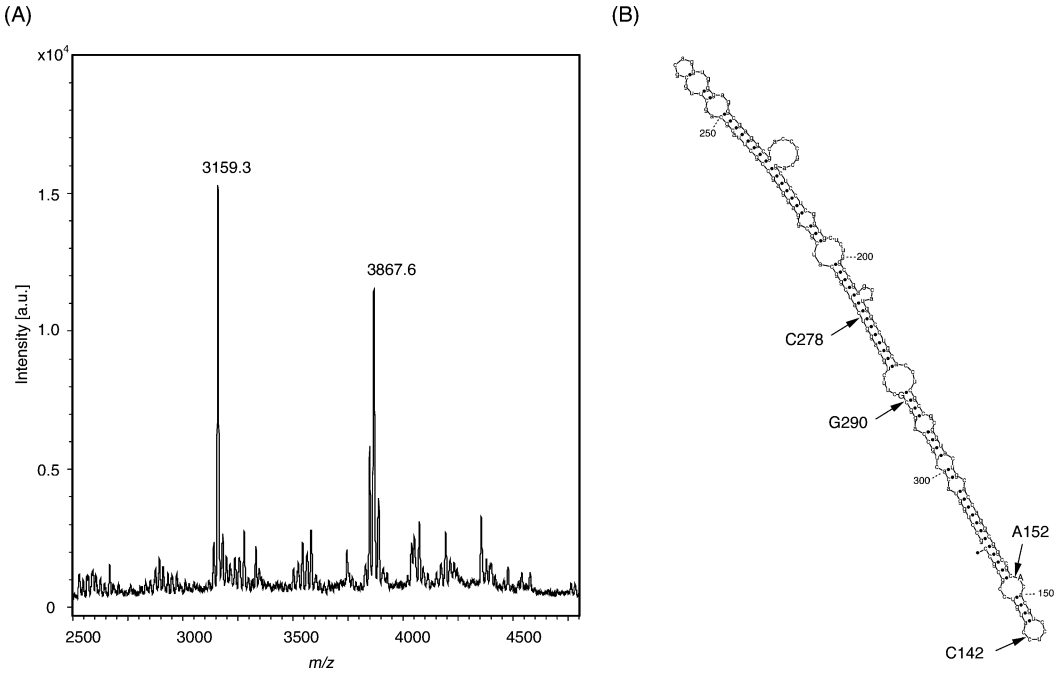

Figure 8. MALDI-TOF MS spectrum for the product of site-selective scission of 179-mer exon RNA from the human *apoE* 3 gene in the presence of both D_1X_{2d} and D_2X_2 (A). The secondary structure of the RNA substrate is shown in (B), where the four scission sites are designated by the arrows. The signal at 3867.6 is for the 12-mer fragment (G290−U279), and that at 3159.3 is for the 10-mer fragment (A152−C143). Other minor peaks are the fragments produced by random hydrolysis of the portions that were free from hybridization with the two-site activators. The measurement was made in positive ion mode. Reaction conditions: pH 7.5 and 25 °C for 24 h; $[\text{substrate RNA}]_0 = 4.0$, [each activator] = 10.0, and [Lu(III)] = 150 μM; [NaCl] = 200 mM.

potential SNP sites (position 152) is A, whereas the nucleotide at position 290 is G.

This 179-mer RNA was treated with Lu(III) ion in the presence of two two-site activators (D_1X_{2d} and D_2X_2). The reaction conditions were exactly the same as those used for the multiplex analysis in Figure 7. As shown by the arrows in Figure 8B, one of the four target scission sites (C142) is located in the hairpin loop, another (C278) in the stem region, and the other two (A152 and G290) in the loop−stem junction. About 75% of the nucleotides, which are recognized by the activators, form base pairs in the secondary structure. Even under these circumstances, all of these target sites were precisely and efficiently hydrolyzed, and the two desired fragments were obtained (see the MS spectrum in Figure 8A). The signal at $m/z = 3159.3$ is from the A152−C143 portion, and that at 3867.6 is for the G290−U279 portion. Both of these m/z values are in fairly good accordance with the calculated numbers ($[M + H]^+ = 3159.5$ and 3867.5, respectively). These two activators functioned almost independently from each other here as observed above. The intensities of other signals from the RNA substrate were much smaller. Apparently, the portions that were free from the hybridization with the two-site activators were randomly hydrolyzed by Lu(III) ion.

Conclusions

By combining an oligonucleotide bearing two acridines (two-site activator) and a lanthanide(III) ion (molecular scissors), RNA cutters for selective scission of two predetermined sites in substrate RNA have been prepared. In these two-site activators, the acridines are incorporated in exchange for the nucleotides. The molecular scissors are simply added to the reaction mixtures without being covalently bound to any sequence-recognizing moieties. In the heteroduplexes between the RNA substrate and the two-site activators, the two phosphodiester linkages in front of the two acridines are significantly activated and hydrolyzed by the lanthanide ions, although these molecular scissors are not immobilized anywhere. Accordingly, the RNA fragments obtained are always longer by one base than the DNA portion between the two acridines in the oligonucleotides. The activations of RNA by the two acridines in the activators occur almost independently from each other. The present two-site scission requires no specific sequence around the scission sites and is sufficiently fast irrespective of the sequence. RNA substrates forming higher-order structures are also acceptable.

As an example of the applications of the present findings, SNPs in both homozygous and heterozygous samples are genotyped by analyzing the fragments with MALDI-TOF MS. One of the two acridines is placed in front of the SNP site. The genotype is conclusively pinned down in terms of the difference in mass numbers of fragments. The present method is also applicable to multiplex analyses, where a number of SNPs are simultaneously analyzed. The length of the fragment from each of the SNP sites can be easily and precisely controllable by changing the number of nucleotides between the acridines in the corresponding activator. Thus, the overlapping of the MS signals from many SNP sites can be minimized, facilitating the simultaneous analysis of the mixtures of many fragments by MALDI-TOF MS. The two-site RNA cutters developed here should be also useful for the preparation of designated fragments from tRNA, ribosomal RNA, virus RNA, and others, thus paving the way to new RNA science.

Experimental Section

Preparation of Oligonucleotides. Reagents were purchased from Glen Research Co. Automated syntheses of DNA and RNA were performed on an ABI 394 DNA synthesizer. Acridine[10] and FAM were introduced to oligonucleotides by using the corresponding phosphor-

amidite monomer. The attachment of TMR to the 3′-end of oligonucleotides was achieved with the CPG column designed for this purpose. Acridine-modified oligonucleotides were removed from the support and deprotected by using 0.4 M sodium hydroxide in 4:1 methanol−water mixture. Crude products were purified by Poly-Pak II cartridges (Glen Research), 20% denaturing PAGE, and then by a reversed-phase HPLC equipped with RP-C18 column (Cica-Merck LiChroCART 125-4: a linear gradient of 5−25% acetonitrile in 0.05 M ammonium formate over 20 min, flow rate 0.5 mL/min). In RNA synthesis, the products were first treated with concentrated aqueous ammonia and then with 1 M tetra-n-butylammonium fluoride in THF. After the mixtures were desalted by NAP-10 column, the RNA was purified by 20% denaturing PAGE and finally by a reversed-phase HPLC. All of these products were characterized by MALDI-TOF MS.

RNA Hydrolyses. The substrate RNA and the complementary oligonucleotides bearing one or two acridine moieties were dissolved in 10 mM Tris-HCl buffer (pH 7.5) containing NaCl (200 mM). The mixture was heated to 90 °C (for 1 min) and slowly cooled to room temperature. Then aqueous solution of $LuCl_3$ (or other lanthanide trichlorides) was added to the mixture (the final concentration, 150 μM). The reaction volume was 10 μL. The reaction was achieved at 25 °C (unless otherwise noted). At higher temperatures, the reactions were faster but slightly less selective.

Polyacrylamide Gel Electrophoresis. After a predetermined reaction time, the reaction was quenched by 100 mM EDTA-2Na solution and analyzed on a 20% denaturing PAGE. The intensities of bands were determined by using the fluorescence from either FAM (excitation at 473 nm) or TMR (excitation at 532 nm). Imaging and quantification were carried out on a Fuji film FLA-3000G fluorescent imaging analyzer.

MALDI-TOF MS. Mass analyses were carried out on a KRATOS Kompact MALDI 2 TOF-MS spectrometer in negative ion mode for synthetic model RNA substrates and on a Bruker AutoFLEX mass spectrometer in positive ion mode for the genomic substrate. Prior to the analyses, the reaction mixtures were desalted by micropipet tip containing C18 media at the end (ZipTip from Millipore). In a typical example, about 6 pmol of RNA fragments could be obtained after this procedure. The mixtures were added to a matrix solution containing 3-hydroxypicolinic acid, 0.5 M diammonium hydrogen citrate, and acetonitrile (30%) and then spotted on a TOF-MS plate. The mass number presented here is associated with an experimental error of 0.05%.

In Vitro Transcription of Genomic RNA Substrate. The DNA fragment of *apoE* 3 exon from nucleotide position 120 to 330 in the human *apoE* gene was amplified by PCR and inserted into pGEM-T easy vectors (Promega). Confirmation of the structure of the construct was made by DNA sequencing and restriction mapping. After cloning, the portion between C131 and G309 was amplified by PCR with an SP6 promoter-appended primer. The required 179-mer RNA was prepared by in vitro transcription with a RiboMAX SP6 RNA transcription kit (Promega) and was purified with an RNeasy mini kit (QIAGEN, Germany). The full sequence of the substrate, as well as its secondary structure calculated by *mfold*, is shown in the Supporting Information.

Measurement of Melting Temperature (T_m). The melting profiles of the duplexes were obtained on a JASCO V-530 spectrometer at 260 nm in a quartz cell of 1-cm path length. The specimens contained RNA fragment (1 μM), the oligonucleotide bearing acridine (1 μM), and NaCl (200 mM) in pH 8 Tris-HCl buffer (10 mM). The heating rate was 1.0 °C/min.

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Supporting Information Available: MALDI-TOF MS spectrum for the product of site-selective scission of R_1G by Lu(III) in the presence of D_1X_{2b}, site-selective activation and hydrolysis of RNA containing an abasic site, and full sequence and the secondary structure of the 179-mer RNA (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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