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Site-selective RNA scission at two sites for precise genotyping of SNPs by mass spectrometry†‡

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Short RNA fragments containing single nucleotide polymorphism (SNP) sites have been selectively clipped out of substrate RNA by using complementary DNA having two acridine residues and Lu^{III}, and the genotype of the substrate is accurately and easily determined by mass analysis of these fragments.

High-throughput, accurate, and low-cost methods for genotyping of single nucleotide polymorphism (SNP) are crucially important for design of tailor-made medicines, prediction of hereditary diseases, and many other biomedical applications.[1,2] Among a number of methods hitherto proposed, mass spectrometry (MS) is one of the most promising ones, since SNP alleles are discriminated in terms of differences in mass number.[3] At present, primer extension methods are widely used to prepare samples for mass analyses.[4] Here we present a new approach for the preparation of mass samples, in which short RNA fragments of desired length and site are clipped out of the RNA substrate by hydrolyzing two designated phosphodiester linkages. It is based on our recent finding of site-selective RNA scission that involves no covalent fixation of catalysts to the sequence-recognizing moiety.[5] By non-covalent interactions of the RNA with an oligonucleotide bearing an acridine, the phosphodiester linkage in front of the acridine is selectively activated and hydrolyzed by various catalysts (*e.g.*, lanthanide(III), Zn^{II}, and Mn^{II} ions). Advantageously, it requires no specific base sequence at the scission site. Thus, two-site RNA scission for genotyping by MS should be possible with the use of an oligonucleotide bearing two acridines, if (1) these acridines do not interfere with each other in RNA activation, (2) the site-selective scissions at these two sites occur without any significant cross talk, and (3) the RNA fragments are sufficiently protected from the subsequent digestion. As described below, all these requirements can be satisfactorily fulfilled by appropriate molecular design. In this study, model RNA substrates coding the exon of human apolipoprotein E gene (APOE) are precisely genotyped by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). The SNPs in this gene (A → G alternation at the nucleotide position 152 in its antisense strand and G → A alternation at the nucleotide position 290)[6] are responsible for Alzheimer's disease and hyperlipemia, respectively.[7,8]

In Fig. 1, RNA_{290}-G (the G309-A271 portion of G-allele in APOE) is treated with $LuCl_3$ in the presence of DNA_{290}-C which has two acridine groups in front of U298 and U285 (the term C refers to the use of conventional nucleoside C opposite the SNP site; *vide infra*).§ The potential SNP site G290 (in bold) is in this sequence. As shown in (b), the MALDI-TOF mass spectrum of the products clearly exhibits a peak at *m/z* = 4168.8, which is in reasonable accord with the theoretical value (4169.5) for the 13-mer fragment from U298 to C286.[9] Both of the target sites are efficiently activated by the acridines and hydrolyzed by Lu^{III}. Moreover, the 13-mer RNA fragment is successfully protected from hydrolysis by Lu^{III}, by forming a heteroduplex with the complementary part in the DNA.[10] These facts have been further substantiated by gel electrophoresis in which the substrate RNA is labeled with tetramethylrhodamine at the 3′-end and with fluorescein at the 5′-end (see ESI†). The scission efficiency at each of the designated sites is similar to the value accomplished by the oligonucleotide bearing one acridine. In a similar fashion, 12-, 14-, 16-mer RNA fragments were also selectively prepared by using DNAs in which two acridines are placed at the corresponding distances (data not shown). The scission sites can be freely chosen. Other lanthanide(III) ions (*e.g.* La^{III} and Eu^{III}) are also applicable.

The present genotyping method is applicable also to heterozygous samples. In such samples, the RNA from the subject involves two components that differ from each other in only one nucleobase, corresponding to the two alleles on homologous chromosomes. The 1 : 1 mixture of RNA_{152}-G and

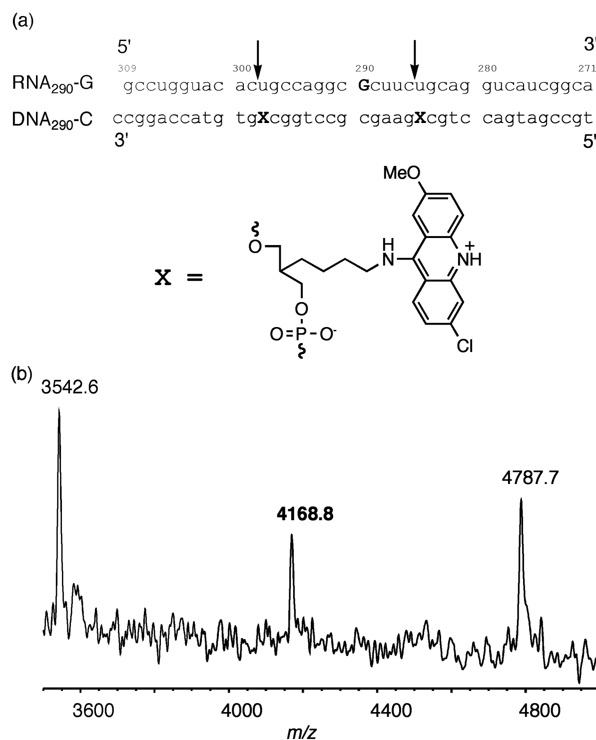

Fig. 1 (a) The oligonucleotides and the X residue bearing an acridine. The two target sites for scission are indicated by arrows. The structure of X has not yet been optimized. (b) Mass spectrum of the cleavage products of RNA_{290}-G in the presence of DNA_{290}-C. The signal at 4168.8 corresponds to the 13-mer fragment (U298–C286) which involves the SNP site G290. The signals at 3542.6 and 4787.7 are for the 5′-side fragment (G309–C299) and the 3′-side fragment (U285–A271), respectively. Reaction conditions: 37 °C for 6 h.

† Electronic supplementary information (ESI) available: 1: confirmation of two-site RNA scission; 2: simultaneous analysis of the two SNP sites in APOE. See http://www.rsc.org/suppdata/cc/b3/b300368j/

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RNA_{152}-A was taken as a typical heterozygous sample, and treated with a combination of Lu^{III} and DNA_{152}-P (Fig. 2(a)). Note that the DNA used here involves non-natural nucleoside dP in front of G152 and A152. This nucleoside analogue notably makes base pairs both with G and with A through its tautomerism.[11] Accordingly, even when there exist two different substrates, both fragments can be obtained in a sufficient amount, since they are strongly bound to DNA_{152}-P and protected from the subsequent hydrolysis. As expected, two signals of similar intensity were detected at m/z 4510.0 and 4494.1, which correspond to the 14-mer fragment from RNA_{152}-G (theoretical m/z = 4512.6) and the one from RNA_{152}-A (4496.7), respectively (Fig. 2(b)). By using DNA_{290}-P, also the 1:1 mixture of RNA_{290}-A and RNA_{290}-G could be analyzed. Quite significantly, when the conventional nucleoside C was used instead of dP (DNA_{152}-C), only the target fragment from RNA_{152}-G was detected and the one from RNA_{152}-A was not obtainable. The degenerate recognition of both A and G by dP is essential for precise genotyping of heterozygous samples.

Another important advantage of the present method is the ease of multiplex analysis, in which multiple substrates are simultaneously processed. A 1:1 mixture of RNA_{290}-G and RNA_{152}-A, which are from two different parts of APOE, was analyzed after being hydrolyzed by Lu^{III} in the presence of both DNA_{290}-P and DNA_{152}-P. For these two DNAs, different lengths between the two acridines were intentionally used, in order to produce two RNA fragments of different lengths and to facilitate mass analysis. As presented in the ESI†, the 13-mer fragment from the corresponding part in RNA_{290}-G (m/z = 4168.2) and the 14-mer fragment from RNA_{152}-A (4494.2) were clearly detected (the theoretical m/z values are 4169.5 and 4496.7, respectively). Here, neither the G → A alternation at position 290 nor the A → G alternation at position 152 exists.

In conclusion, short RNA fragments of desired length and site have been prepared by combining oligonucleotides bearing two acridines and lanthanide(III) ion. By using these RNA fragments, SNP sites in both homozygous and heterozygous samples could be precisely genotyped by MALDI-TOF MS. For clinical applications, *in vitro* transcripts from amplified genomic DNA should be prospective substrates. Moreover, the present strategy should be applicable also to various other purposes such as RNA processing and structural studies. Such applications, as well as studies to improve sensitivity and cleavage activity, are currently underway in our laboratory.

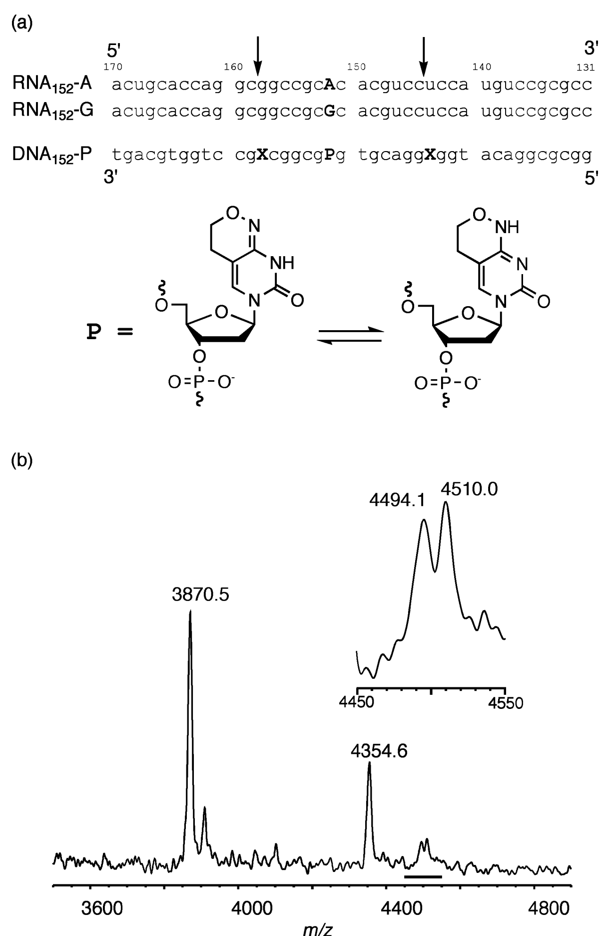

Fig. 2 (a) The oligonucleotides and nucleoside analogue dP. (b) Mass spectrum of the cleavage products from a 1:1 mixture of RNA_{152}-A and RNA_{152}-G in the presence of DNA_{152}-P. The inset shows the magnification of the region between m/z 4450.0 and 4550.0. The signals at 4494.1 and 4510.0 are for the 14-mer fragments (G158–C145) from RNA_{152}-A and from RNA_{152}-G, respectively. The signals at 3870.5 and 4354.6 are for the 5′-side fragment (A170–C159) and the 3′-side fragment (U144–C131), respectively. Reaction conditions: 25 °C for 24 h.

Notes and references

§ For the RNA cleavage, the total concentration of RNA was 4.0 μM, and the concentration of each DNA was 10 μM ([$LuCl_3$] = 150 μM, [Tris-HCl] = 10 mM, and [NaCl] = 200 mM; pH 7.5; total volume, 10 μL). After a predetermined time, the reaction mixture was desalted by a micropipette tip containing C18 media at the end (ZipTip® from Millipore Co.), and directly used for analysis on a KRATOS Kompact MALDI 2 TOF-MS spectrometer. The mass number is associated with an experimental error of 0.05%. All the oligonucleotides were synthesized with standard phosphoramidite chemistry, purified by PAGE and HPLC, and characterized by MALDI-TOF MS.

1 A. J. Brookes, *Gene*, 1999, **234**, 177.
2 (*a*) A. C. Syvänen, *Nature Rev. Genet.*, 2001, **2**, 930; (*b*) B. W. Kirk, M. Feinsod, R. Favis, R. M. Kliman and F. Barany, *Nucleic Acids Res.*, 2002, **30**, 3295; (*c*) V. Lyamichev, A. L. Mast, J. G. Hall, J. R. Prudent, M. W. Kaiser, T. Takova, R. W. Kwiatkowski, T. J. Sander, M. de Arruda, D. A. Arco, B. P. Neri and M. A. D. Brow, *Nature Biotechnol.*, 1999, **17**, 292; (*d*) A. Alderborn, A. Kristofferson and U. Hammerling, *Genome Res.*, 2000, **10**, 1249.
3 (*a*) D. Lechner, G. M. Lathrop and I. G. Gut, *Curr. Opin. Chem. Biol.*, 2002, **6**, 31; (*b*) T. J. Griffin and L. M. Smith, *Trends Biotechnol.*, 2000, **18**, 77; (*c*) P. Ross, L. Hall, I. Smirnov and L. Haff, *Nature Biotechnol.*, 1998, **16**, 1347; (*d*) K. Nakai, W. Habano, T. Fujita, K. Nakai, J. Schnackenberg, K. Kawazoe, A. Suwabe and C. Itoh, *Hum. Mutat.*, 2002, **20**, 133; (*e*) S. Kim, J. R. Edwards, L. Y. Deng, W. Chung and J. Y. Ju, *Nucleic Acids Res.*, 2002, **30**, e85; (*f*) S. Sauer, D. H. Gelfand, F. Boussicault, K. Bauer, F. Reichert and I. G. Gut, *Nucleic Acids Res.*, 2002, **30**, e22; (*g*) S. Zhang, C. K. Van Pelt and G. A. Schultz, *Anal. Chem.*, 2001, **73**, 2117; (*h*) A. Mattes and O. Seitz, *Angew. Chem., Int. Ed.*, 2001, **40**, 3178; (*i*) T. J. Griffin, J. G. Hall, J. R. Prudent and L. M. Smith, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 6301; (*j*) P. L. Ross, K. Lee and P. Belgrader, *Anal. Chem.*, 1997, **69**, 4197.
4 For efficient genotyping of SNPs by MALDI-TOF MS, 10- to 20-mer fragments are generally desirable.
5 (*a*) A. Kuzuya, R. Mizoguchi, F. Morisawa, K. Machida and M. Komiyama, *J. Am. Chem. Soc.*, 2002, **124**, 6887; (*b*) A. Kuzuya, K. Machida, R. Mizoguchi and M. Komiyama, *Bioconj. Chem.*, 2002, **13**, 365.
6 The numbering is based on that in the sense DNA strand, and thus increases from the 3′-end of RNA to the 5′-end.
7 D. A. Nickerson, S. L. Taylor, S. M. Fullerton, K. M. Weiss, A. G. Clark, J. H. Stengård, V. Salomaa, E. Boerwinkle and C. F. Sing, *Genome Res.*, 2000, **10**, 1532.
8 C. M. Vanduijn, P. Deknijff, M. Cruts, A. Wehnert, L. M. Havekes, A. Hofman and C. Vanbroeckhoven, *Nature Genet.*, 1994, **7**, 74.
9 The site-selective scission predominantly occurs at the 5′-side of the nucleotide in front of the acridine and produces 3′- (or 2′-) monophosphate (see ref. 5). Since there are 12-nucleotides between the acridines in this DNA, the 13-mer RNA fragment is selectively formed.
10 Compared with single-stranded RNA, the RNA in duplexes is far more resistant to the hydrolysis by lanthanide(III) ions: K. A. Kolasa, J. R. Morrow and A. P. Sharma, *Inorg. Chem.*, 1993, **32**, 3983.
11 P. K. T. Lin and D. M. Brown, *Nucleic Acids Res.*, 1989, **17**, 10373.