Targeted Site-Specific Gas-Phase Cleavage of Oligoribonucleotides. Application in Mass Spectrometry-Based Identification of Ligand Binding Sites

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Mass spectrometry-based methods have demonstrated utility in the characterization of oligonucleotides and their noncovalent complexes.[1−4] The dissociation of deoxy- and ribonucleic acids via infrared multiphoton or collisionally activated dissociation (CAD) produces a mixture of fragment ions, and the base sequence can be reconstructed from measurement of their masses.[5−8] For longer oligonucleotides ($n \geq 20$), the abundance of fragment ions is an uneven function of base sequence and position along the chain.[8]

The fragmentation of oligonucleotides is a complex process, but appears related to the relative strengths of the glycosidic bonds.[9−11] We exploit this observation by incorporating deoxynucleotides selectively into a chimeric 2′-*O*-methylribonucleotide model of the bacterial rRNA A site region.[12] During CAD, fragmentation is directed to the more labile deoxynucleotide sites. The resulting CAD mass spectrum contains a small subset of readily assigned complementary fragment ions. Binding of ligands near the deoxyadenosine residues inhibits the CAD process, while complexation at remote sites does not affect dissociation and merely shifts the masses of specific fragment ions. These methods are used to identify compounds from a combinatorial library that preferentially bind to the RNA model of the A site region.

The 27mer model of a segment of the bacterial A site region has been prepared as a full ribonucleotide (Figure 1, compound **R**), and as a chimeric 2′-*O*-methylribonucleotide containing three deoxyadenosine residues (Figure 1, compound **C**).[13] The aminoglycoside paromomycin binds to both **R** and **C** with K_D values of 0.25 and 0.45 μM, respectively.[14] Paromomycin has been shown previously to bind in the major groove of the 27mer model RNA and induce a conformational change, with contacts to A_{1408}, G_{1494}, and G_{1491}.[12]

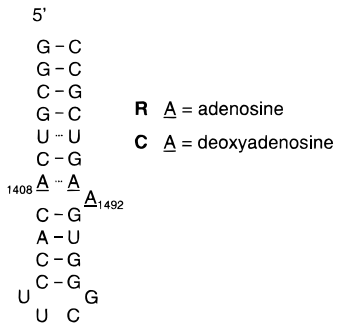

Figure 1. Sequences and structures for oligonucleotides **R** and **C**. The underlined nucleotides in **C** are deoxyadenosines, while the remainder of the nucleotides are 2′-*O*-methyl nucleotides. The numbering is relative to 16S ribosomal RNA.

The mass spectrum obtained from a 5 μM solution of **C** mixed with 125 nM paromomycin (Figure 2a) contains $[M\text{-}5H]^{5-}$ ions from free **C** at *m*/*z* 1783.6 and the $[M\text{-}5H]^{5-}$ ions of the paromomycin−**C** complex at *m*/*z* 1907.3.[15] Signals from the $[M\text{-}4H]^{4-}$ ions of **C** and the complex are detected at *m*/*z* 2229.8 and 2384.4, respectively. No signals are observed from more highly charged ions as observed for samples denatured with tripropylamine. In analogy with studies of native and denatured proteins, this is consistent with a more compact structure for **C** and the paromomycin complex. The CAD mass spectrum obtained from the $[M\text{-}5H]^{5-}$ ion of **C** is presented in Figure 2b. Fragment ions are detected at *m*/*z* 1005.6 $(w_6)^{2-}$, 1065.8 $(a_7\text{-}B)^{2-}$, 1162.6 $(w_7)^{2-}$, 1756.5 $(M\text{-}Ad)^{5-}$, 2108.9 $(w_{21}\text{-}Ad)^{3-}$, 2153.4 $(a_{20}\text{-}B)^{3-}$, 2217.8 $(w_{21})^{3-}$, and 2258.3 $(a_{21}\text{-}B)^{3-}$.[16] These fragment ions all result from loss of adenine from the three deoxyadenosine nucleotides, followed by cleavage of the 3′-C−O sugar bonds. The CAD mass spectrum for the $[M\text{-}5H]^{5-}$ ion of the complex between **C** and paromomycin obtained with the same activation energy is shown in Figure 2c. No fragment ions are detected from strand cleavage at the deoxyadenosine sites using identical dissociation conditions of Figure 2b. The change in fragmentation pattern observed upon binding of paromomycin is consistent with a change in the local charge distribution, conformation, or mobility

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(13) RNAs **R** and **C** have been prepared using conventional phosphoramidite chemistry on solid support. Phosphoramidites were purchased from Glen Research and used as 0.1 M solutions in acetonitrile. RNA **R** was prepared following the procedure given in Wincott, F.; Usman, N.; *Nucl. Acids Res*. **1995**, *23*, 2677−2684. RNA **C** was prepared using standard coupling cycles, deprotected, and precipitated from 10 M NH_4OAc.
(14) The reported K_D values are ∼0.2 μM: (a) Recht, M. I.; Fourmy, D.; Blanchard, S. C.; Dahlquist, K. D.; Puglisi, J. D.; *J. Mol. Biol*. **1996**, *262*, 421−436. (b) Wong, C. H.; Hendrix, M.; Priestley, E. S.; Greenberg, W. A.; *Chem. Biol*. **1998**, *5*, 397−406. (c) Wang, Y.; Hamasaki, Y.; Rando, R. R.; *Biochemistry*, **1997**, *36*, 768−779.
(15) Mass spectrometry experiments have been performed on an LCQ quadrupole ion trap mass spectrometer (Finnigan; San Jose, CA) operating in the negative ionization mode. RNA and ligand were dissolved in a 150 mM ammonium acetate buffer at pH 7.0 with isopropyl alcohol added (1:1 v:v) to assist the desolvation process. Parent ions have been isolated with a 1.5 *m*/*z* window, and the AC voltage applied to the end caps was increased until ∼70% of the parent ion dissociates. The electrospray needle voltage was adjusted to −3.5 kV, and spray was stabilized with a gas pressure of 50 psi (60:40 N_2:O_2). The capillary interface was heated to a temperature of 180 °C. The He gas pressure in the ion trap was 1 mTorr. In MS-MS experiments, ions within a 1.5 Da window having the desired *m*/*z* were selected via resonance ejection and stored with $q = 0.2$. The excitation RF voltage was applied to the end caps for 30 ms and increased manually to 1.1 V_{pp} to minimize the intensity of the parent ion and to generate the highest abundance of fragment ions. A total of 128 scans were summed over *m*/*z* 700−2700 following trapping for 100 ms.
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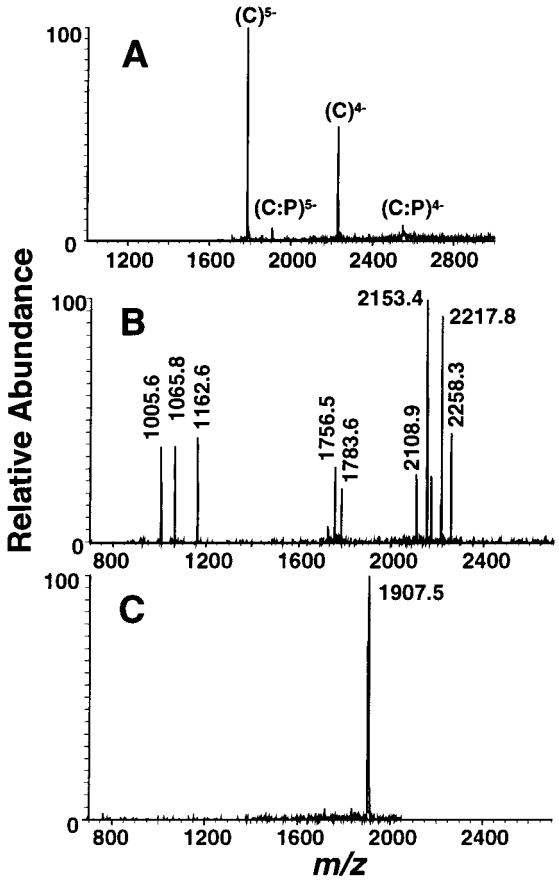

Figure 2. (a) Mass spectrum obtained from a mixture of 5 μM **C** and 125 nM paromomycin (**P**).[15] (b) MS-MS spectrum obtained following isolation of $[M\text{-}5H]^{5-}$ ions (*m*/*z* 1783.6) from uncomplexed **C**. (c) MS-MS spectrum obtained following isolation of $[M\text{-}5H]^{5-}$ ions (*m*/*z* 1907.5) from **C** complexed with paromomycin.

of A1492, A1493, and A1408 that precludes collisional activation and dissociation of the nucleotide.

Two combinatorial libraries containing 216 tetraazacyclophanes dissolved in DMSO were mixed with a buffered solution containing 10 μM **C** such that each library member is present at 100 nM.[17] The resulting mass spectra reveal >10 complexes between **C** and library members with the same nominal mass. Ions from the most abundant complex from the first library ($[M\text{-}5H]^{5-}$ *m*/*z* 1919.0) have been isolated and dissociated. As shown in Figure 3a, dissociation of this complex generates three fragment ions at *m*/*z* 1006.1, 1065.6, and 1162.4 that result from cleavage at each dA residue. More intense signals are observed at *m*/*z* 2378.9, 2443.1, and 2483.1. These ions correspond to the $w_{21}^{(3-)}$, $a_{20}-B^{(3-)}$, and $a_{21}-B^{(3-)}$ fragments bound to a library member with a mass of 676.0 $\pm$ 0.6 Da. The relative abundances of the fragment ions are similar to the pattern observed for uncomplexed **C**, but the masses of the ions from the lower stem and tetraloop are shifted by complexation with the ligand. This ligand offers little protection of the deoxyadenosine residues, and must bind to the lower stem-loop. The library did not inhibit growth of bacteria.[17] Dissociation of the most abundant complex from a mixture of **C** and the second library having *m*/*z* 1934.3 *with the same collisional energy* (Figure 3b) yields few fragment ions, the predominant signals arising from intact complex and loss of neutral adenine. The mass of the ligand (753.5 Da) is consistent with six possible compounds in the library having two combinations of functional groups. The reduced level of cleavage and loss of adenine for this complex is consistent with binding of the ligand at the model A site region as does paromomycin. The second library inhibits transcription/translation at 5 μM, and has an MIC of 2−20 μM against *E. coli(imp-)* and *S. pyogenes*.[17]

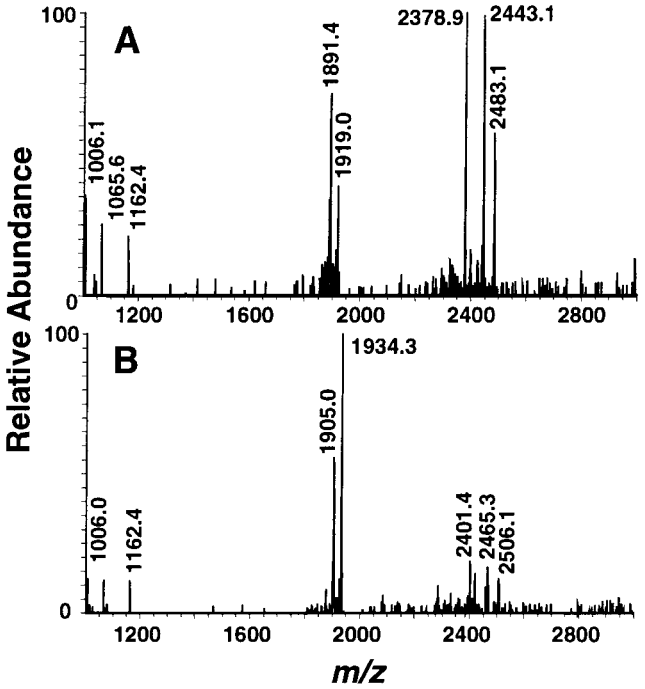

Figure 3. MS-MS spectra obtained from a mixture of 10 μM **C** and a 216 member combinatorial library (a) following isolation of $[M\text{-}5H]^{5-}$ ions (*m*/*z* 1919.0) from **C** complexed with ligand(s) of mass 676.0 $\pm$ 0.6 Da and (b) following isolation of $[M\text{-}5H]^{5-}$ ions (*m*/*z* 1934.3) from **C** complexed with ligand(s) of mass 753.5 $\pm$ 0.6 Da. Ion isolation and dissociation conditions are identical with those described in Figure 2, except 512 scans were summed in sets of eight.

Mass spectrometry-based assays provide many advantages for identification of complexes between RNA and small molecules. All constituents in the assay mixture carry an intrinsic mass label, and no additional modifications with radioactive or fluorescent tags are required to detect the formation of complexes. The chemical composition of the ligand can be ascertained from the measured molecular mass of the complex, allowing rapid deconvolution of libraries to identify leads against an RNA target. Incorporation of deoxynucleotides into a chimeric oligoribonucleotide generates a series of labile sites where collisionally activated dissociation is favored. Binding of ligands at the labile sites affords protection from CAD observed in MS-MS experiments. This mass spectrometry-based protection assay can be used to establish the binding sites for small molecule ligands without the need for additional chemical reagents or radiolabeling of the RNA. The methodology may have applications in DNA sequencing and identification of genomic defects.[10]

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