Sequence-Specific Cleavage of HIV mRNA by a Ribozyme Mimic

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We report here the preparation and activity of a functional mimic of ribozymes. Ribozymes are catalytic RNA molecules that promote a variety of reactions, including the hydrolytic cleavage of RNA and DNA.[1,2] This relatively new class of enzymes requires divalent metal ions for two distinct purposes. Metal ions play a structural role (assuring the proper folding of the ribozyme's RNA backbone to achieve an active structure) and a catalytic chemical role (stabilizing the transition state, activating the substrate and/or stabilizing the leaving group). The ribozyme sequence and its metal cofactors combine to provide substrate recognition, proper active-site folding, and chemical reactivity. As recently pointed out by Pyle,[3] the simultaneous structural and chemical behavior of metal ions in ribozymes complicates the development of a precise understanding of either function.

We have designed *functional* mimics of ribozymes in which the position and coordination environment of the catalytic metal ion can be controlled through chemical synthesis. This novel approach allows the separation of catalytic activity and recognition roles into separate structural domains and is designed to provide an improved understanding of the chemical role that metal ions play in ribozyme activity. We first identified metal complexes that cleave RNA hydrolytically[4] and then incorporated these complexes into oligodeoxynucleotides (ODNs).[5,6] The ODN provides molecular recognition for a specific target RNA sequence, and the covalently-attached metal complex provides cleavage activity. This design allows us to control activity by changing the metal, its coordination number and ligand set, and the length and conformational flexibility of the linker arm that joins the cleavage and recognition domains. In a parallel study, we have developed DNA building blocks with attached organic catalysts such as histidine, imidazole, and polyamines.[7]

We previously reported that (terpyridyl)$Cu^{II}OH^+$ (**1**) is effective at both the transesterification[4a] and hydrolysis[4a,b] of RNA under physiologically relevant conditions (pH = 7, 37 °C). We therefore designed and synthesized a derivative of **1** that was suitable for incorporation into a DNA probe. The elaboration of **1** into a DNA building block is shown in Figure 1. Terpyridine derivative **2**[6,8] was deprotected with hydrazine to give amine **3**,

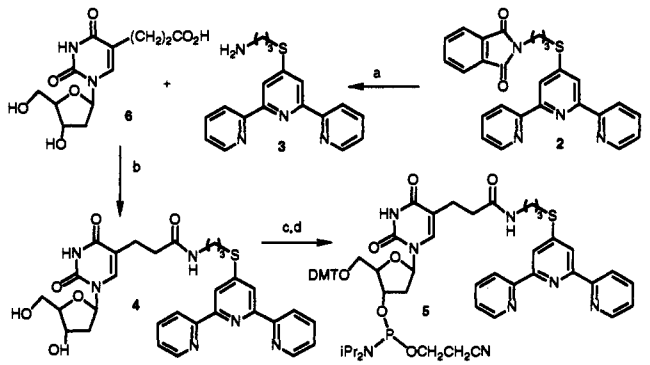

Figure 1. (a) Hydrazine, EtOH, 60 °C, yield 96%; (b) ethyl[3-(dimethylamino)propyl]carbodiimide hydrochloride, DMSO, room temperature, 24 h, yield 77%; (c) pyridine, DMTCl, 24 h, yield 65%; (d) iPr$_2$NEt, THF, 4 °C, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, yield 86%.

1(775)
5'-GGAGAAAUUU AUAAAAGAUG GAUAAUCCUG GGAUUAAAUA
AAAUAGUAAG AAUGUAUAGC CCUACCAGCA UUCUGGACAU
AAGACAAGGA CCAAAGGAAC **CCUUUAGAGA CUAUGUAG**AC
CGGUUCUAUA AAACUCUAAG AGCCGAGCAA GCUUCACAG-3'
159(933)

Figure 2. Sequence of the RNA target. The ribozyme mimic (oligo X) and control (oligo T) are complementary to the boldface region.

which was then coupled to a 2'-deoxyuridine derivative.[7a,9] This molecular design affords thymidine analogue **4**, where the terpyridine assembly takes the place of a thymidine methyl group. Functionalization at the C-5 methyl position was employed[9] because derivatives of this type are competent at Watson–Crick base pairing, with minimal interference in hydrogen bonding. CPK molecular models and modeling with Quanta showed that a Cu^{II} hydroxide bound to the terpyridyl group of **4** can reach across the major groove of an A-form DNA–RNA duplex and attack the opposite strand. Nucleoside **4** was converted into phosphoramidite **5** for use in solid-phase DNA synthesis.[10]

Our initial target was a 159-mer RNA sequence derived from a conserved region of the *gag*-mRNA of HIV (HIVHXB2r), corresponding to nucleotides 775–933. The RNA target, whose sequence is shown in Figure 2, was prepared by transcription from a DNA template using T7 RNA polymerase.[11] For sequence-specific cleavage reactions and control studies, we prepared two 17-mer ODNs complementary to the boldface region of the target (Figure 2). The ribozyme mimic, 5'-pCTA CAX AGT CTC TAA AG-3' (oligo X), was prepared using phosphoramidite **5**, where **X** indicates the terpyridine-containing nucleotide. This modified nucleotide is complementary to A113 of the RNA target. The control sequence (oligo T) contains T in place of **X**.

Cleavage reactions were carried out with 5'[^{32}P]-end-labeled RNA target at a concentration of ca. 10^{-8} M. The products were separated by gel electrophoresis under denaturing conditions. Results of control experiments and sequence-specific cleavage reactions are shown in Figure 3. No specific cleavage was seen in the absence of oligo X (lanes 1–4). Lane 5 shows that the combination of Cu^{2+} ion and oligo X cleaved the RNA at two specific sites. The extent of specific cleavage was 11% at 37 °C (data not shown) and 18–25% at 45 °C. The sites of hydrolytic

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(10) Data for **5**: ^{31}P NMR (121.4 MHz, CD_3CN) δ 149.59, 149.62 ppm (ref: H_3PO_4[ext]); FAB HRMS (M + H)$^+$ = 1107.5, calcd for $C_{60}H_{68}N_8O_9$-SP 1107.4568. Purification: basic alumina/5–7% MeOH in CH_2Cl_2.
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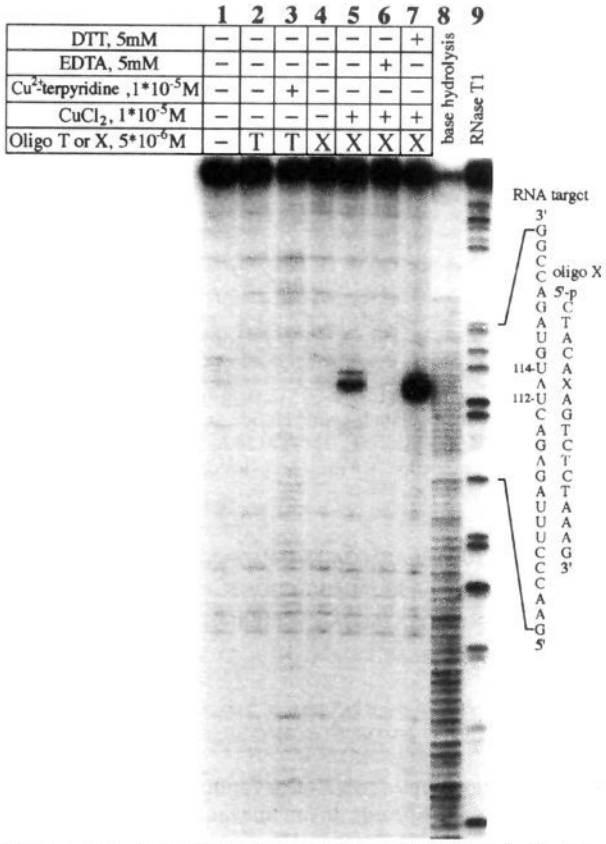

Figure 3. Cleavage of the 159-mer RNA target by oligo X. Reaction mixtures contained 5′[^{32}P]-end-labeled RNA (ca. 10 nM), 20 mM HEPES–NaOH (pH 7.5), and 0.1 M $NaClO_4$. As indicated, RNA was incubated in the presence (+) or absence (–) of oligo T, oligo X, $CuCl_2$, complex **1**, DTT, and EDTA. ODN concentrations were 5×10^{-6} M, Cu(II) concentrations were 1×10^{-5} M, and ODNs were premixed with Cu(II) before addition to the RNA. All reactions were carried out for 72 h at 45 °C and were analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea. Control lane 1 shows the intact RNA target. Oligo T did not cleave the RNA sequence-specifically, in either the presence or absence of aqueous (terpyridyl)Cu^{II} (lanes 2 and 3). Oligo X alone did not cleave the target (lane 4), but oligo X plus Cu^{2+} cleaved the RNA in two specific sites (lane 5). Cleavage was suppressed by EDTA (lane 6). Oxidative cleavage of six-base region was initiated by DTT (lane 7). The identity of product bands was confirmed by comparison with lane 8 (alkaline hydrolysis) and lane 9 (RNase T1 digestion). The autoradiogram was quantified with a Molecular Dynamics densitometer.

cleavage are shown in Figure 4 (top arrows): the X-nucleotide hydrogen bonds to A113, and cleavage occurs predominantly between U112 and A113.

We have previously shown that, in the absence of added reducing agents, **1** cleaves RNA hydrolytically but does not cleave DNA.[4a,5a] This chemoselectivity is important in our design of ribozyme

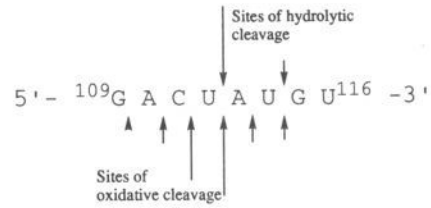

Figure 4. Sites of hydrolytic and oxidative cleavage. The target region from G109 to U116 is shown. Note that oxidative cleavage was at least 2–2.5 times more efficient than hydrolytic cleavage under our conditions.

mimics. However, **1** can be driven to perform redox cleavage of both RNA and DNA by the addition of reducing agents such as dithiothreitol (DTT). To compare the hydrolytic and oxidative reactions of oligo X, RNA cleavage was studied in the presence of DTT. As shown in Figure 3, lane 7, this resulted in a dramatic increase in cleavage efficiency (to 43%) and a complete change in the reaction products: instead of predominant reaction at U112, densitometry showed that cleavage occurred at six major positions, from G109 to U114. Fitting of the densitometry results to Gaussian peaks gave the identity and relative abundance of the major oxidative cleavage products, as depicted in Figure 4. A higher resolution gel showing sequence-specific cleavage of a 20-mer DNA target by oligo X confirmed this analysis: DNA was not cleaved unless DTT was added (data not shown).

Our design of ribozyme mimics is based on the concept that a reagent capable of hydrolytic RNA cleavage can be incorporated into an oligonucleotide or oligonucleotide analog, thereby combining reactive and molecular recognition domains within one synthetic construct. Sequence-specific cleavage of the RNA target was achieved by oligo X, *within the duplex region.* Since cleavage within the duplex lowers the RNA–DNA binding constant, this strategy may allow product release and catalytic turnover to be achieved.[12] The new class of enzyme mimic reported here allows independent control of (a) the active-site metal and its coordination environment,[13] (b) the distance between reactive center and recognition element, and (c) the level of complementarity between the target and probe. Ribozyme mimics may have utility in explaining the role of metal ions in natural ribozymes, in RNA structure mapping, as RNA endonculeases, and as *catalytic antisense drugs* for damaging injurious genomic or messenger RNA.

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