Communication

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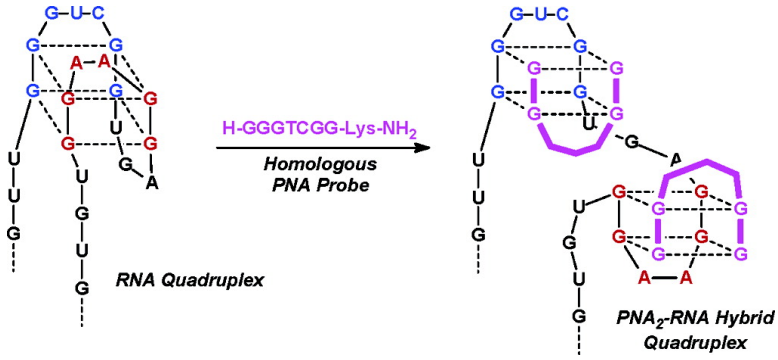
J. Am. Chem. Soc., **2005**, 127 (22), 8032-8033• DOI: 10.1021/ja051102y • Publication Date (Web): 14 May 2005



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Published on Web 05/14/2005

RNA Guanine Quadruplex Invasion by Complementary and Homologous PNA Probes

Violeta L. Marin and Bruce A. Armitage*

Department of Chemistry, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213

Received February 21, 2005; E-mail: army@andrew.cmu.edu

DNA and RNA oligomers that contain multiple runs of consecutive guanine (G) nucleotides are capable of folding into stable secondary structures known as G-quadruplexes, wherein four Gs are hydrogen bonded together into a roughly square planar array.[1] Stacking of these tetrads and coordination of metal ions at the center of the structure yield a G-quadruplex.[2] This secondary structural motif is gaining increasing attention as evidence grows implicating G-quadruplexes in regulation of gene expression. For example, a DNA G-quadruplex is suspected to form upstream of the main promoter for the *c-MYC* oncogene and to function as a repressor for transcription.[3] Meanwhile, an RNA G-quadruplex is believed to regulate alternative splicing of the pre-mRNA coding for hTERT, the reverse transcriptase component of the enzyme telomerase.[4] Thus, synthetic compounds that bind to G-quadruplexes could find significant applications as therapeutics or as probes for gene function.

This communication describes the use of peptide nucleic acid (PNA) to invade guanine-rich sequences of RNA that fold into quadruplex structures. Peptide nucleic acid (PNA) is a DNA analogue in which the negatively charged phosphate backbone of DNA is replaced with an achiral, uncharged polyamide backbone formed by repetitive units of *N*-(2-aminoethyl) glycine units to which the nucleobases are attached via an acetyl linker.[5] Due to the lack of electrostatic repulsion between the uncharged PNA and the polyanionic target, PNA hybridizes with high affinity and sequence specificity to complementary DNA and RNA targets according to the Watson−Crick base pairing rules.[6] Prior work has shown that PNA molecules bind to complementary sequences within DNA quadruplex targets with high affinity.[7,8] More recently, we demonstrated that a homologous PNA (i.e., a PNA having the *same* sequence as the target) disrupts a bimolecular DNA G-quadruplex to form a stable PNA_2−DNA_2 hybrid quadruplex.[9] Herein, we demonstrate that the complementary and homologous recognition of quadruplexes by PNA can be extended to RNA targets, as well, specifically targeting a unimolecular quadruplex.

The target for these experiments was **RDQ** (Chart 1), an RNA aptamer originally selected for binding to the Fragile X mental retardation protein.[10] ^{1}H NMR spectroscopy supported the proposed secondary structure, which consists of distinct duplex and quadruplex domains.[11] The results of UV melting experiments are also consistent with the proposed structure. A particularly useful wavelength for monitoring melting transitions is 295 nm because G-quadruplexes exhibit a *hypochromic* transition at this wavelength, while duplex transitions are *hyperchromic*.[12] Figure 1 illustrates the results for melting of **RDQ** in the presence of 100 mM KCl. A low-temperature hypochromic transition is observed, followed by a hyperchromic transition at higher temperature. This suggests that the quadruplex domain in **RDQ** unfolds at lower temperature, followed by opening of the remaining RNA hairpin by disruption of the base-paired stem. This interpretation is supported by the fact that the lower transition is not observed when LiCl replaces KCl,

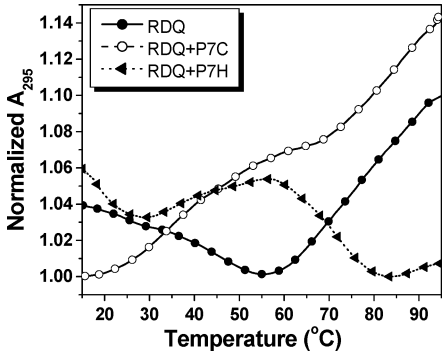
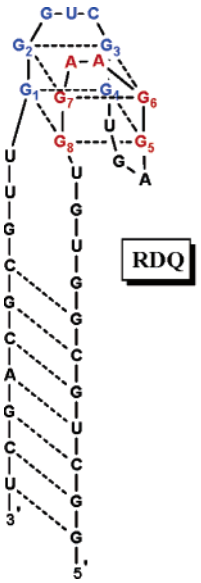


Figure 1. UV melting curves for RNA quadruplex alone and with complementary or homologous PNA probes; 2 μM samples were prepared in buffer containing 10 mM Tris/HCl (pH = 7.0) and 100 mM KCl.

Chart 1. RNA Target, PNA Probes, and Thiazole Orange Label



Complementary PNA Probes

P7C H-CCCAGCC-Lys-NH_2

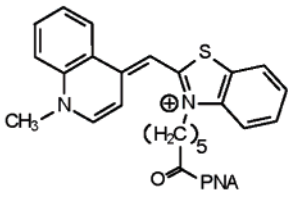
P7C$_{TO}$ TO-CCCAGCC-Lys-NH_2

Homologous G-rich PNA Probes

P7H H-GGGTCGG-Lys-NH_2

P7H$_{TO}$ TO-GGGTCGG-Lys-NH_2

Thiazole Orange (TO)



while the upper transition is unaffected (Supporting Information, Figure S1). G-quadruplexes exhibit specific ion requirements, with potassium strongly preferred over lithium.[13]

PNA probe **P7C** is complementary to the region of **RDQ** shown in blue (Chart 1). Successful hybridization of the PNA requires "invasion" of the quadruplex domain in order to form a stable duplex. The melting curve recorded for a 1:1 mixture of **P7C** and **RDQ** is shown in Figure 1 and clearly shows that the hypochromic G-quadruplex melting transition observed for **RDQ** alone is replaced by a hyperchromic transition which we assign to melting of the 7 bp PNA−RNA duplex. In contrast, the upper transition due to melting of the RNA duplex region is still observed.

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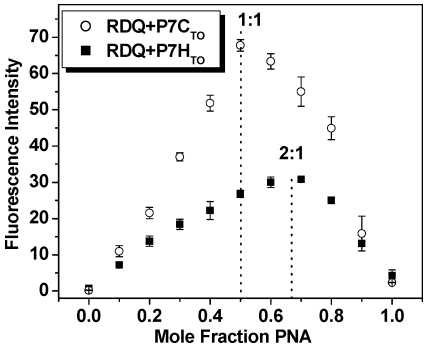

Figure 2. Job plot for complementary and homologous PNAs binding to **RDQ** at a total concentration of 0.5 μM monitored by fluorescence. Samples were prepared in 10 mM Tris/HCl (pH = 7.0) with 100 mM KCl and were annealed prior to recording the spectra (λ_{Ex} = 495 nm).

CD spectroscopy is commonly used to detect hybridization reactions, but very little difference was observed in the spectra recorded for **RDQ** in the absence and presence of **P7C** (data not shown). This is due to the fact that the CD spectrum of **RDQ** is very similar to the CD spectrum previously reported for a PNA−RNA duplex.[6] Further support for successful hybridization of **P7C** was obtained from fluorescence experiments using a PNA probe end-labeled with a thiazole orange (TO) derivative. TO is essentially nonfluorescent in fluid solution but becomes strongly fluorescent when placed in a rigid environment.[14] Svanvik, Kubista, and coworkers demonstrated that TO−PNA conjugates exhibit enhanced fluorescence upon hybridization to complementary DNA strands, most likely due either to end-stacking of the fluorophore onto the PNA−DNA duplex or to partial intercalation into DNA nucleobases extending beyond the hybrid region.[15] Figure S2 illustrates fluorescence spectra recorded for **P7C$_{TO}$** in solution and in the presence of **RDQ**. The fluorescence increases approximately 40-fold upon hybridization to the RNA. This was used to determine an equilibrium binding constant for PNA hybridization of 8.1×10^8 M^{-1} (Figure S3).

We next synthesized PNA probe **P7H** to test for hybrid PNA−RNA quadruplex formation. This PNA is homologous to the same target site as the complementary probe **P7C**. The melting curve recorded for a 1:1 mixture of the homologous PNA and **RDQ** is shown in Figure 1 (triangles). In contrast to the complementary probe, a hypochromic transition is still observed in the presence of **P7H**. However, this transition occurs at much higher temperature (ΔT_m = ca. 30 °C), and the transition assigned to melting of the RNA duplex region is not observed. Substituting LiCl for KCl in the solution causes the T_m to decrease by approximately 30 °C, and the hyperchromic transition due to melting of the RNA stem reappears (Figure S4). These results indicate that the **P7H** forms a hybrid quadruplex with **RDQ**.

A TO-labeled version of **P7H** demonstrated a 5-fold fluorescence enhancement in the presence of **RDQ** (Figure S5). Given the polymorphic nature of G-quadruplex structures in DNA, it was not immediately obvious how many PNA strands would hybridize to the intramolecular G-quadruplex in **RDQ**. Figure 2 shows that the complementary PNA forms a 1:1 hybrid, as expected based on the constraints of Watson−Crick base pairing. However, the homologous PNA clearly forms a 2:1 complex, meaning two **P7H$_{TO}$** strands bind to a single **RDQ**. It is important to note that while **P7H** is perfectly homologous to the 7 nucleotide target site shown in Chart 1, hybridization should be mediated by the guanine bases that participate in G-tetrad formation while the central non-G bases are less important. Thus, **P7H** is "G-homologous" to **RDQ** in several ways, depending on which two G-tracts it uses to form the hybrid quadruplex. For example, if one **P7H** hybridizes to the blue target region in Chart 1 (i.e., G_1-G_2 and G_3-G_4), then the two G-tracts displaced from the **RDQ** quadruplex (i.e., G_5-G_6 and G_7-G_8, shown in red in Chart 1) would be available for hybridizing with a second homologous PNA, with the only mismatches arising from the linkers joining the two G-tracts in the probe and target. The implications of this degeneracy on the sequence selectivity of homologous hybridization will be the subject of a future investigation.

These relatively short PNA probes overcome kinetic and thermodynamic obstacles to hybridization presented by the folded secondary structure of **RDQ**. Hybridization occurs within 10 min at room temperature and submicromolar PNA and RNA concentrations. The thermodynamic driving force for quadruplex invasion remains to be determined, although both duplex and quadruplex hybrids should involve more hydrogen bonding and π-stacking interactions than are present in the RNA quadruplex. Electrostatic interactions are likely favorable, as well, since hybridization will result in Coulombic attraction between the cationic PNA and the RNA target, while also relieving intrastrand electrostatic repulsions due to opening of the RNA quadruplex.

In conclusion, both complementary and homologous PNA probes invade an intramolecular RNA quadruplex and form stable PNA−RNA duplex and quadruplex structures, respectively.

Acknowledgment. We thank the National Institutes of Health (RO1 GM58547) and Carnegie Mellon University for financial support of this research, Dr. Babu Rao Renikuntla for synthesis of the thiazole orange derivative, and Laurel Grotzinger for expert technical assistance. V.M. thanks the CMU Department of Chemistry for a Harrison Legacy Dissertation Fellowship. MALDI-TOF mass spectra were recorded in the Center for Molecular Analysis at Carnegie Mellon University, supported by NSF Grants CHE-9808188 and DBI-9729351.

Supporting Information Available: UV melting curves, fluorescence spectra, and binding constant determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA051102Y