JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Binding of a Structured D-RNA Molecule by an L-RNA Aptamer

Jonathan T. Sczepanski and Gerald F. Joyce*

Departments of Chemistry and Molecular Biology and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, United States

Supporting Information

ABSTRACT: An L-RNA aptamer was developed that binds the natural D-form of the HIV-1 trans-activation responsive (TAR) RNA. The aptamer initially was obtained as a D-aptamer against L-TAR RNA through *in vitro* selection. Then the corresponding L-aptamer was prepared by chemical synthesis and used to bind the desired target. The L-aptamer binds D-TAR RNA with a K_d of 100 nM. It binds D-TAR exclusively at the six-nucleotide distal loop, but does so through tertiary interactions rather than simple Watson−Crick pairing. This complex is the first example of two nucleic acids molecules of opposing chirality that interact through a mode of binding other than primary structure. Binding of the L-aptamer to D-TAR RNA inhibits formation of the Tat-TAR ribonucleoprotein complex that is essential for TAR function. This suggests that L-aptamers, which are intrinsically resistant to degradation by ribonucleases, might be pursued as an alternative to antisense oligonucleotides to target structured RNAs of biological or therapeutic interest.

Noncoding RNAs are increasingly being recognized as playing important roles in biology, especially in the regulation of gene expression.[1] This has prompted efforts to inhibit such RNAs in a target-specific manner, both to investigate their biological function and to develop potential therapeutic agents.[2] The simplest approach for the target-specific inhibition of RNA is antisense technology, employing complementary oligodeoxynucleotides. For most applications it is preferable to use oligonucleotide analogues that are resistant to degradation by nucleases, for example, 2′-*O*-methyl oligonucleotides or "locked" nucleic acids. In principle the specificity of these compounds is assured by the specificity of Watson−Crick (WC) pairing, but in practice there often are off-target effects due to partial complementarity of the antisense agent to other RNAs.[3]

A different strategy for target-specific inhibition of RNA is the aptamer approach, employing *in vitro* selection methods to discover nucleic acid molecules that bind to the target with high affinity. This strategy has two important limitations. First, the resulting aptamer must be modified to confer nuclease resistance, although some protein-binding aptamers have been developed starting from populations of nuclease-resistant oligonucleotide analogues,[4] and in principle the same could be done for aptamers that bind RNA. The second limitation is the tyranny of WC pairing—the strong tendency to recognize RNA through complementary interactions—which applies to both standard oligonucleotides and their nuclease-resistant analogues. WC pairing is the most expedient way to bind RNA and is difficult to outcompete with a more nuanced mode of recognition.

L-RNA, the enantiomer of natural D-RNA, is incapable of binding D-RNA through WC pairing and is completely resistant to degradation by nucleases.[5] Several small-molecule- and protein-binding L-RNA aptamers have been developed,[6] including compounds that are currently undergoing human clinical trials.[7] However, L-RNA aptamers have never been reported for D-RNA targets. Perhaps this is because antisense is such an obvious, albeit imperfect, solution to the binding problem. In addition, the early literature incorrectly suggested that L-RNA can hybridize to D-RNA through WC pairing in a parallel orientation.[5,8] That suggestion prompted efforts to develop L-RNA and L-DNA as antisense agents,[9] which were abandoned when it became clear that stable duplexes do not form between nucleic acids of opposing chirality.[5,10] With an eye toward the many noncoding RNAs that one might target through specific tertiary interactions rather than WC pairing, efforts to develop L-RNA aptamers against D-RNA targets seemed overdue.

L-Aptamers (usually referred to as "Spiegelmers")[6a] are initially selected as D-aptamers against the enantiomer of the target ligand, enabling enzymatic amplification of the D-RNA during the process of *in vitro* selection.[11] Then the corresponding L-RNA is prepared by chemical synthesis and used to bind the desired target. Protein-binding L-aptamers, for example, typically are obtained by selecting D-RNAs that bind a D-peptide corresponding to the enantiomer of a structural epitope within the target protein. The same methodology could be applied to structured RNA targets, provided the target RNA can be prepared as the L-stereoisomer. The increasing use of L-aptamers and antiviral agents derived from L-ribose has made L-nucleoside phosphoramidites readily available, which are required to prepare L-RNA by solid-phase synthesis.[12] Thus, a variety of short (<100 nucleotides (nt's)), biologically relevant target RNAs, such as microRNAs, riboswitches, and even portions of the ribosome, can be obtained through chemical synthesis, allowing aptamers to be selected against them. This study reports the first example of an L-aptamer selected to bind a structured D-RNA target.

The target RNA chosen for this study is the trans-activation responsive (TAR) element of HIV-1 RNA. This RNA has an extended stem-loop structure that is crucial for efficient transcription of the integrated genome of HIV-1.[13] TAR RNA is an attractive target due to its well-characterized structure and function.[14] In addition, both D-RNA and D-DNA aptamers have previously been selected against this target.[15] These aptamers were shown to bind TAR RNA through a "kissing hairpin"

Received: June 29, 2013
Published: August 26, 2013

ACS Publications © 2013 American Chemical Society

dx.doi.org/10.1021/ja406634g | *J. Am. Chem. Soc.* 2013, 135, 13290−13293

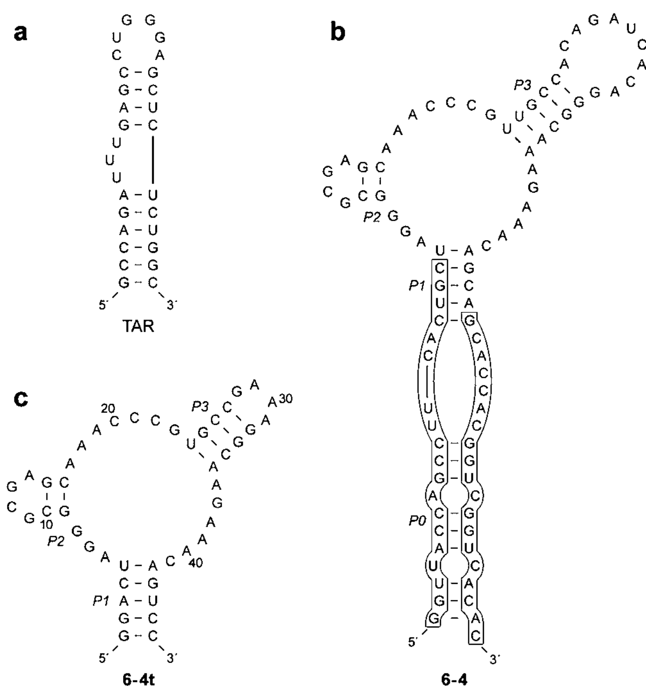

Figure 1. Sequence and secondary structure of TAR RNA and an anti-TAR RNA aptamer of opposing chirality. (a) 29-nt RNA corresponding to the distal stem-loop of TAR, encompassing the site of Tat binding. (b) Aptamer clone **6-4**, with boxed regions indicating the fixed primer-binding sites. (c) Truncated aptamer **6-4t**, containing 46 nt's.

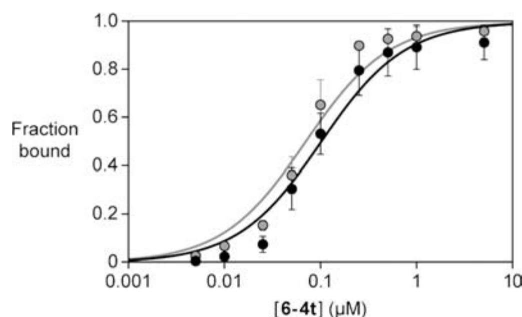

Figure 2. Saturation plot for binding of either D-TAR and L-**6-4t** (black) or L-TAR and D-**6-4t** (gray). Data were obtained in triplicate and fit to the equation: F_{bound} = [**6-4t**]/(K_d + [**6-4t**]).

complex involving a loop that is fully complementary to the distal loop of the TAR hairpin (with the sequence 5′-CUGGGA-3′). Such an interaction could not be realized between RNAs of opposing chirality.[5,10] Thus L-aptamers are expected to bind D-TAR RNA through tertiary interactions rather than WC pairing.

In vitro selection was carried out using a 29-nt truncated version of TAR RNA (Figure 1a, see Supporting Information (SI)), chemically synthesized using commercially available L-nucleoside phosphoramidites. A pool of ~10^{14} D-RNA candidates, each containing 50 random-sequence nt's flanked by fixed primer-binding sites (see SI), was mixed with 5′-biotinylated L-TAR RNA at room temperature in the presence of 10 mM $MgCl_2$, 50 mM NaCl, 0.1% TWEEN 20, and 25 mM Tris (pH 7.6). D-RNAs that bound L-TAR were captured using magnetic streptavidin beads. The beads were washed several times with the same binding solution, and aptamers that remained bound then were eluted using 25 mM NaOH, reverse transcribed, and amplified by PCR. The resulting dsDNAs were used to transcribe a corresponding pool of RNAs to begin the next round of *in vitro* selection. A total of six rounds were carried out, gradually increasing the stringency of selection by decreasing the concentration of pool RNA and lengthening the washing period (see SI). Following the sixth round, the amplified DNA was cloned and sequenced.

Of 21 clones that were analyzed, D-**6-4** (Figure 1b), which occurred five times, had an apparent K_d for L-TAR RNA of <1 μM when measured by an electrophoretic mobility-shift assay (EMSA). Thus, all further studies were conducted using this clone. A series of truncations of D-**6-4** were made to establish the minimal D-RNA sequence that is required to bind L-TAR. Based on these studies, a secondary structural model of D-**6-4** was established, involving three paired elements (P0−P1, P2, and P3) that converge on a central unpaired region (Figure 1b). The P0 portion of the P0−P1 stem, containing the two primer binding sites, could be deleted without disrupting binding of L-TAR RNA (see SI). In fact, a truncated version of D-**6-4** that lacked these nt's had greater affinity for L-TAR compared to that of the parent aptamer. In addition, replacement of the 11-nt loop that closes the P3 stem with a simple tetraloop (5′-GAAA-3′) had little effect on binding. Together, these observations were used to design a minimal aptamer, D-**6-4t**, that contains only 46 nt's (Figure 1c). The sequence of the P1 stem of D-**6-4t** was modified to install a 5′-terminal G residue, required for *in vitro* transcription using T7 RNA polymerase, and the P3 stem was shortened by one base pair. These changes did not have an appreciable effect on binding, consistent with the predicted secondary structure of D-**6-4t**. Interestingly, a version of D-**6-4t** that contains no uridine residues (which occur only within the paired regions) retains the ability to bind L-TAR with only slightly reduced affinity (see SI).

The affinity of D-**6-4t** and L-**6-4t** for L-TAR and D-TAR, respectively, was determined using chemically synthesized materials. Both aptamers were prepared by solid-phase RNA synthesis using 2′-*tert*-butyldimethylsilyl (TBDMS) RNA phosphoramidites. The 2′-triisopropylsilyloxymethyl (TOM) protecting group is preferred for the chemical synthesis of long RNAs, but is not commercially available in the L series. Thus, to maintain parity with the L-RNA molecules synthesized using TBDMS phosphoramidites, D-**6-4t** was synthesized in the same manner. As expected, the K_d values of D-**6-4t** for L-TAR and of L-**6-4t** for D-TAR are very similar (70 ± 10 and 100 ± 30 nM, respectively; Figure 2). *In vitro* transcribed D-**6-4t** binds L-TAR RNA with a K_d of 30 nM, lower than that for either synthetic complex. This reflects the somewhat lower quality of materials obtained by chemical synthesis compared to enzymatic synthesis of a 46-nt RNA, consistent with previous observations.[16] Not surprisingly, neither D- nor L-**6-4t** was able to bind TAR-RNA of the same chirality (see SI).

To gain insight into how **6-4t** binds TAR RNA, a series of partial hydrolysis experiments were carried out, comparing these molecules in isolation and within the bound complex. Structured RNA elements, including paired regions, typically show reduced susceptibility to hydrolytic cleavage due to disfavored in-line attack of the 2′-hydroxyl on the vicinal phosphate.[17] In contrast, unstructured or heterogeneously structured regions tend to be more susceptible to hydrolysis. Reactions were carried out under mildly alkaline conditions (pH 8.5) to accelerate RNA hydrolysis. This increase in pH relative to the selection conditions (pH 7.6) did not affect the K_d of the aptamer-TAR RNA complex (see SI). Incubation of D-TAR RNA in the presence of a saturating concentration of L-**6-4t** resulted in

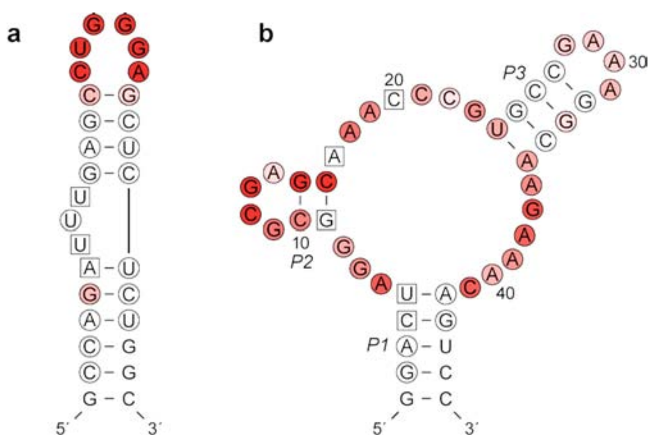

Figure 3. Protection of nucleotide positions against hydrolytic cleavage in the aptamer-TAR complex compared to either molecule in isolation. Increasing red intensity corresponds to increasing levels of protection. (a) D-TAR protected by saturating L-**6-4t**. (b) D-**6-4t** protected by saturating L-TAR. Boxes indicate nt positions having increased susceptibility to hydrolysis in the aptamer-TAR complex.

significant protection of the distal loop of D-TAR RNA compared to the absence of L-**6-4t** (Figure 3a). In contrast, hydrolytic cleavage of the internal UUU-bulge was enhanced by the presence of the aptamer. This suggests that L-**6-4t** interacts with D-TAR RNA largely through the 6-nt distal loop. In support of this conclusion, L-**6-4t** was found to bind several other short D-RNA hairpins that contained a different stem sequence, so long as they contained the distal loop associated with TAR RNA (see SI).

Incubation of free D-**6-4t** under the same conditions as above revealed a cleavage pattern consistent with the predicted secondary structure shown in Figure 1c. For example, phosphodiester linkages in the loop that closes the P3 stem (5′-GAAA-3′) are more susceptible to cleavage than are those within the adjacent stem. Cleavage at most sites within the central unpaired region of D-**6-4t** was significantly reduced in the presence of saturating concentrations of L-TAR RNA (Figure 3b). This indicates that adaptive binding of the ligand creates a defined RNA tertiary structure that impedes hydrolytic cleavage in this region. Cleavage at positions within the P1 and P3 stems of D-**6-4t** was unaffected by the presence of L-TAR RNA, indicating that these structural elements are not effected by TAR binding and further supporting the predicted secondary structure of D-**6-4t**. Several positions within D-**6-4t** exhibited increased hydrolysis in the presence of L-TAR (Figure 3b), suggesting that these positions are made more flexible upon binding of L-TAR. Taken together, the partial hydrolysis data suggest that D-**6-4t** binds L-TAR RNA through tertiary interactions involving unpaired nt's within the aptamer core and the distal loop of TAR RNA. There is no WC complementarity, in either the parallel or antiparallel sense, between these two regions. Various nt's within the central unpaired region of D-**6-4t** were mutated, in each case resulting in the complete loss of L-TAR binding (see SI), again consistent with tertiary interactions rather than WC pairing between the aptamer core and the distal loop of TAR.

Because L-RNA aptamers are resistant to degradation by nucleases, they might be used to target and thus block the function of structured RNAs of biological interest. To demonstrate this, L-**6-4t** was tested for its ability to inhibit binding of D-TAR RNA by the HIV-1 transactivator protein (Tat). The Tat-TAR interaction is crucial for efficient transcription of HIV-1 mRNA. Thus, several TAR-binding agents, including a D-RNA aptamer, have been developed to block formation of the Tat-TAR complex.[18] The binding sites for the Tat protein and L-**6-4t** on D-TAR RNA do not overlap. Tat recognizes the uridine bulge and adjacent nt's within the stem,[19] whereas the aptamer binds the 6-nt distal loop. Nonetheless, L-**6-4t** was expected to inhibit Tat binding based on the protein's sensitivity to the conformation of the distal loop and previously observed inhibition of Tat binding by a D-DNA aptamer that binds at this location.[20] In addition, hydrolytic cleavage within the uridine bulge of TAR was increased in the presence compared to absence of the aptamer (Figure 3a), suggesting altered geometry in this region.

Inhibition by L-**6-4t** of the Tat-TAR interaction was examined by EMSA using the full-length D-TAR hairpin, prepared by *in vitro* transcription. Because formation of the Tat-TAR complex is inhibited by high ionic strength solutions, it was necessary to conduct the inhibition experiments under conditions of lower ionic strength (3 mM $MgCl_2$, 20 mM KCl), compared to the conditions that were employed during *in vitro* selection (10 mM $MgCl_2$, 50 mM NaCl). The L-**6-4t** aptamer has somewhat reduced affinity for D-TAR RNA under the low-salt conditions (K_d = 160 nM), but still strongly inhibits the Tat-TAR interaction (Figure 4). Adding 2.5 μM L-**6-4t** almost completely

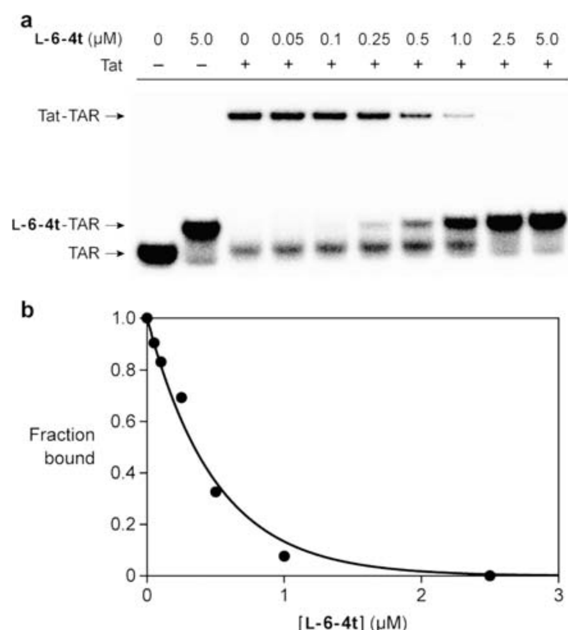

Figure 4. Inhibition of Tat-TAR complex formation by L-**6-4t**, as determined by mobility shift in a non-denaturing polyacrylamide gel. (a) In the absence of Tat, a saturating concentration of L-**6-4t** results in reduced mobility of [5′-^{32}P]-labeled TAR. Adding 1 μM Tat results in formation of the Tat-TAR complex, which is competed by increasing concentrations of L-**6-4t**. (b) Desaturation of the Tat-TAR complex in the presence of increasing concentrations of L-**6-4t**.

blocked formation of the Tat-TAR complex. The IC_{50} for this inhibition was ~400 nM. There was no detectable interaction between L-**6-4t** and Tat when both were present at 100 μM each.

A functional assay was carried out to test the ability of L-**6-4t** to inhibit Tat-dependent transcriptional activation of a portion of HIV-1 genomic DNA that includes the TAR element.[21] *In vitro* transcription was carried out in HeLa cell nuclear extracts in either the presence or absence of 2 μM Tat, and either the

presence or absence of 10 μM L-**6-4t** (see SI). Adding Tat resulted in a 3-fold enhancement of transcription yield, which was reduced by 3-fold when the aptamer also was present. The aptamer alone had no effect on transcription yield.

The vast majority of agents that target RNA rely on WC pairing to recognize their target, thus requiring accessibility to unpaired regions with the structured RNA.[22] In contrast, L-**6-4t** binds D-TAR RNA through tertiary interactions, more closely resembling the interactions of RNA and protein. This is likely to be a general phenomenon when L-aptamers are selected to bind D-RNAs due to the unfavorable energetics of duplex formation between D- and L-RNA. In principle, D-aptamers also could bind structured D-RNAs through tertiary interactions. However, given the ease of discovering WC pairing interactions between two RNAs of the same handedness, recognition through primary sequence alone tends to dominate the outcome of such *in vitro* selection experiments. It will be interesting to select L-aptamers against a diverse range of structured D-RNA targets, such as internal loops or hairpins of varying sizes, to determine whether recognition through tertiary interactions is a general phenomenon.

The use of L-aptamers for targeting structured nucleic acids has several potential advantages compared to antisense and antisense-related technologies. Recognition of D-RNA by L-aptamers involves adapting to rather than competing against the structure of the target RNA. A similar approach was taken by Disney et al. in using small molecules to target structured elements within RNA.[23] Small molecules generally have the advantage of greater bioavailability, whereas RNA molecules have the advantage of evolvability. A major shortcoming of RNA is its extreme sensitivity to degradation by ribonucleases, which are ubiquitous in biological materials. However, by employing L-RNA, selected as a D-RNA against the enantiomer of the desired target, complete resistance to nuclease degradation is assured.

Binding of an L-aptamer to a target RNA through tertiary interactions is expected to be more specific than binding through simple WC pairing because the latter is tolerant of mismatches within the region of hybridization, typically involving 10−20 base pairs. Instead the aptamer recognizes structural features of the target RNA that depend on sequence in a more idiosyncratic manner. Greater effort is required to devise enantiomeric aptamers compared to antisense oligonucleotides, but the synthesis of L-RNA and the *in vitro* selection of D-RNA are now routine, so the outcome may justify this modest additional effort.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods; figures showing starting materials, selected clones, and their structural variants and demonstrating binding interactions between various forms of TAR RNA and the selected aptamer; complete refs 2c, 3b, 4b,c, 6c, 7a−c, 8, and 10. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

gjoyce@scripps.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful to Michael Zwick for supplying plasmid pLAI-BS, used in the transcription assays. This work was supported by Grant No. GM065130 from the National Institutes of Health. J.T.S. was supported by Ruth L. Kirschstein National Research Service Award No. F32GM101741 from the National Institutes of Health.

■ REFERENCES

(1) (a) Bartel, D. P. *Cell* **2009**, *136*, 215. (b) Garzon, R.; Calin, G. A.; Croce, C. M. *Annu. Rev. Med.* **2009**, *60*, 161. (c) Castel, S. E.; Martienssen, R. A. *Nat. Rev. Genet.* **2013**, *14*, 100. (d) Sabin, L. R.; Delás, M. J.; Hannon, G. J. *Mol. Cell* **2013**, *49*, 783.

(2) (a) Krützfeldt, J.; Rajewsky, N.; Braich, R.; Rajeev, K. G.; Tuschl, T.; Manoharan, M.; Stoffel, M. *Nature* **2005**, *438*, 685. (b) Choi, W. Y.; Giraldez, A. J.; Schier, A. F. *Science* **2007**, *318*, 271. (c) Elmén, J.; et al. *Nature* **2008**, *452*, 896.

(3) (a) van Dongen, S.; Abreu-Goodger, C.; Enright, A. J. *Nat. Methods* **2008**, *5*, 1023. (b) Lindow, M.; et al. *Nat. Biotechnol.* **2012**, *30*, 920.

(4) (a) Lin, Y.; Qiu, Q.; Gill, S. C.; Jayasena, S. D. *Nucleic Acids Res.* **1994**, *22*, 5229. (b) Ruckman, J.; et al. *J. Biol. Chem.* **1998**, *273*, 20556. (c) Burmeister, P. E.; et al. *Chem. Biol.* **2005**, *12*, 25.

(5) Ashley, G. W. *J. Am. Chem. Soc.* **1992**, *114*, 9731.

(6) (a) Klussmann, S.; Nolte, A.; Bald, R.; Erdmann, V. A.; Furste, J. P. *Nat. Biotechnol.* **1996**, *14*, 1112. (b) Nolte, A.; Klussmann, S.; Bald, R.; Erdmann, V. A.; Furste, J. P. *Nat. Biotechnol.* **1996**, *14*, 1116. (c) Leva, S.; et al. *Chem. Biol.* **2002**, *9*, 351.

(7) (a) Wlotzka, B.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 8898. (b) Helmling, S.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 13174. (c) Darisipudi, M. N.; et al. *Am. J. Pathol.* **2011**, *179*, 116.

(8) Rypniewski, W.; et al. *Acta Crystallogr. D: Biol. Crystallogr.* **2006**, *62*, 659.

(9) (a) Fujimori, S.; Shudo, K.; Hashimoto, Y. *J. Am. Chem. Soc.* **1990**, *112*, 7436. (b) Asseline, U.; Hau, J.-F.; Czernecki, S.; Le Diguarher, T.; Perlat, M.-C.; Valery, J.-M.; Thuong, N. T. *Nucleic Acids Res.* **1991**, *19*, 4067.

(10) Garbesi, A.; et al. *Nucleic Acids Res.* **1993**, *21*, 4159.

(11) Williams, K. P.; Liu, X. H.; Schumacher, T. N. M.; Lin, H. Y.; Ausiello, D. A.; Kim, P. S.; Bartel, D. P. *Proc. Natl Acad. Sci. U.S.A.* **1997**, *94*, 11285.

(12) Okano, K. *Tetrahedron* **2009**, *65*, 1937.

(13) Jeang, K.-T.; Xiao, H.; Rich, E. A. *J. Biol. Chem.* **1999**, *274*, 28837.

(14) Aboul-ela, F.; Karn, J.; Varani, G. *Nucleic Acids Res.* **1996**, *24*, 3974.

(15) (a) Boiziau, C.; Dausse, E.; Yurchenko, L.; Toulme, J.-J. *J. Biol. Chem.* **1999**, *274*, 12730. (b) Ducongé, F.; Toulmé, J.-J. *RNA* **1999**, *5*, 1605.

(16) Olea, C. J.; Horning, D. P.; Joyce, G. F. *J. Am. Chem. Soc.* **2012**, *134*, 8050.

(17) Soukup, G. A.; Breaker, R. R. *RNA* **1999**, *5*, 1308.

(18) Aboul-ela, F.; Karn, J.; Varani, G. *J. Mol. Biol.* **1995**, *253*, 313.

(19) Churcher, M. J.; Lamont, C.; Hamy, F.; Dingwall, C.; Green, S. M.; Lowe, A. D.; Butler, J. G.; Gait, M. J.; Karn, J. *J. Mol. Biol.* **1993**, *230*, 90.

(20) Darfeuille, F.; Arzumanov, A.; Gryaznov, S.; Gait, M. J.; Di Primo, C.; Toulmé, J.-J. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 9709.

(21) (a) Marciniak, R. A.; Calnan, B. J.; Frankel, A. D.; Sharp, P. A. *Cell* **1990**, *63*, 791. (b) Arzumanov, A.; Walsh, A. P.; Liu, X.; Rajwanshi, V. K.; Wengel, J.; Gait, M. J. *Nucleosides Nucleotides Nucl.* **2001**, *20*, 471.

(22) Milner, N.; Mir, K. U.; Southern, E. M. *Nat. Biotechnol.* **1997**, *15*, 537.

(23) Tran, T.; Disney, M. D. *Nat. Commun.* **2012**, *3*, 1125.