Reporter Ribozymes for Real-Time Analysis of Domain-Specific Interactions in Biomolecules: HIV-1 Reverse Transcriptase and the Primer–Template Complex**

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The major task in the postgenome-era is to decipher the function of thousands of new proteins, their involvement in regulatory networks and to check their suitability as pharmaceutical drug targets. For this reason, novel methods are required that facilitate rapid and reliable identification of molecular interactions of complex biological systems compatible with high-throughput screening protocols. Knowledge about the interaction partners, binding affinity, and interacting domains represent the basis for identification of the biological function and discovery of novel inhibitors, modulators, and drug leads of a given protein.[1] Although there are several powerful methods available for detection and quantification of molecular interactions,[2–7] they are often not generally applicable or compatible with high-throughput screening protocols, in real time. Hence, the development of novel, broadly applicable methods, independent of target protein function, is of fundamental importance.

We are interested in using ribozymes for developing functional assays that allow the analysis of interactions of biologically relevant molecules in real-time. We have reported a novel system for rapid and reliable measurement of the catalytic activity of the hammerhead ribozyme (HHR) by using substrate oligonucleotides labeled with two fluorescent dyes.[8] The spatial proximity of the two dyes results in fluorescence quenching of the donor fluorophore by fluorescence resonance energy transfer (FRET). Ribozyme cleavage activity can be then monitored by a time-dependent increase of fluorescence in real-time. By using these reporter ribozymes we have identified novel inhibitors of the hammerhead ribozyme[9] and of the HIV-1 Rev protein,[10] which were also able to inhibit the biological function of the target molecule in vivo.

Herein, we report the rational design of a reporter ribozyme, which is specifically regulated by HIV-1 reverse transcriptase (HIV-1 RT). We demonstrate that the HIV-1 RT dependent reporter ribozyme is not only capable of selectively detecting the presence of HIV-1 RT but also of sensing the domain-specific interaction of other HIV-1 RT binders such as the primer–template complex.

For the construction of the reporter ribozyme we have chosen a strategy similar to that used for previous systems that are regulated by small organic molecules, by inserting an aptamer sequence into stem II of the HHR. Proper folding of stem III is essential for cleavage activity of the HHR.[11–14] We have chosen an aptamer which was selected by Tuerk et al. from a combinatorial RNA-library and which binds HIV-1 RT with an affinity of 25 pM.[15] The crystal structure of the RNA–protein complex shows that the *anti*-HIV-1 RT aptamer in the complex with HIV-1 RT forms a pseudoknot structure, in which the 5′- and 3′-ends of the aptamers are spatially separated.[16]

We have deliberately chosen an aptamer with a pseudoknot structure because this motif is often used as a regulatory element in nature. For example, formation of a pseudoknot induces a frameshift in some viral mRNA sequences.[17] In some eukaryotic transcripts, a pseudoknot structure in the 5′-untranslated region leads to activation of a regulatory protein, which then locally controls translation of the transcript.[18]

Owing to these known structural and regulatory features of pseudoknot motifs the *anti*-HIV-1 RT aptamer seemed to be well suited as a regulatory element of a hammerhead ribozyme. The aptamer was inserted into stem II of the HHR, as shown in Figure 1a, resulting in a fusion construct FK-1 with competing folds of the ribozyme and the pseudoknot structures. The simultaneous folding of both domains is impossible in this design, because in the absence of HIV-1 RT the inserted aptamer sequence folds into a hairpin loop structure (see Figure 1a, left). As shown in Figure 1b, the reporter ribozyme FK-1 is active in the absence of HIV-1 RT due to the folding of the hairpin loop, forming stem II in FK-1. The presence of an unpaired loop was proven by digestion with ribonucleases specific for single-stranded RNA (Figure 2). In the presence of HIV-1 RT, the catalytic activity of FK-1 is inhibited (Figure 1b) due to the induction of the pseudoknot fold by the protein. This leads to disruption of stem II and, hence, to the inhibition of cleavage activity.

To further characterize and verify the influence of the stability of stem II on the capability of structural changes of FK-1, two variants of FK-1 were generated, one with weakened and one with stabilized stem II structures. Deletion of the GC base pair highlighted in gray in Figure 1a yields construct FK-2 with a destabilized stem II (Figure 1c). The complete absence of catalytic activity of FK-2 (Figure 1d) indicates that the lack of the stabilizing GC base pair diminishes formation of the catalytically active conformation in favor of the pseudoknot fold. Indeed, nuclease digest reactions of FK-2 shown in Figure 2 resulted in cleavage patterns, which are in accordance with the kinetic data in Figure 1d, thus supporting the exclusive formation of the pseudoknot.

For further validation of this hypothesis we constructed a third version of the reporter ribozyme, which is only capable of forming the catalytically active fold, but not the pseudoknot structure. Starting from FK-1, an additional GC base pair was inserted, which leads to stabilization of stem II in the construct FK-3 (Figure 1e). Figure 1f shows, as expected, that FK-3 is catalytically active, with the cleavage activity remaining unchanged even in the presence of HIV-1 RT. Owing to the increased stability of stem II, the protein is no longer able to induce the folding of the pseudoknot. Indeed, the nuclease

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

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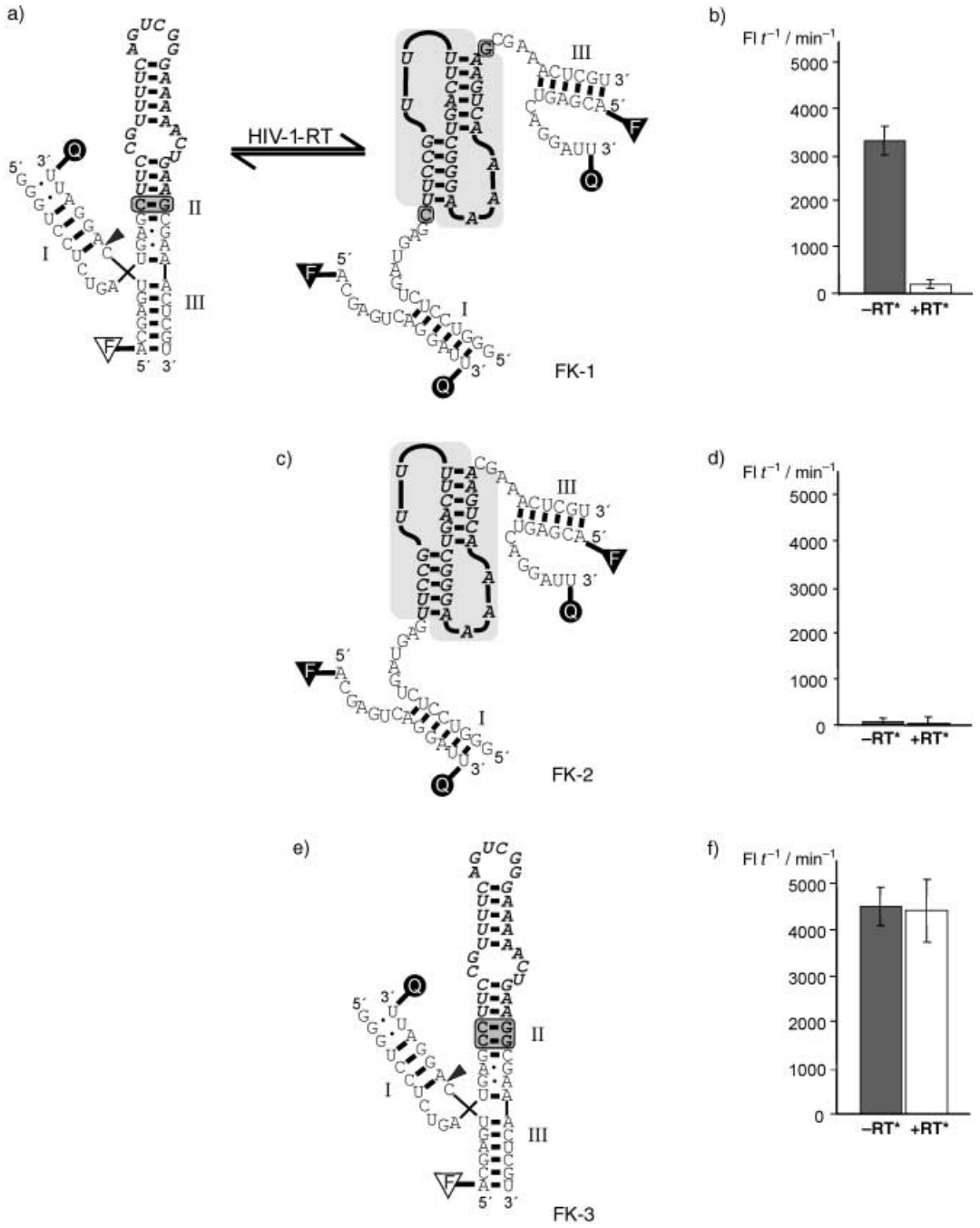

Figure 1. Structures and cleavage activities of the fusion constructs FK-1, FK-2, and FK-3. Aptamer sequences inserted into the hammerhead ribozyme are highlighted in boldface italics. Complexes with one or two FRET-labeled substrates are shown (5′-fluorophore: F, 3′-quencher: Q). a) The fusion of the HIV-1 RT aptamer with a hammerhead ribozyme forms the correct ribozyme fold with stems I, II, and III (left). The arrow indicates the cleavage site. Addition of HIV-1 RT induces the formation of the pseudoknot (highlighted in pale gray), disrupting the formation of stem II (right). b) Cleavage activity of FK-1 in absence and presence of 200 nM HIV-1 RT (RT*). The cleavage rate of a FRET-labeled substrate in the initial 5 min is shown, measured in fluorescence per time interval (Fl t^{-1}/min^{-1}).[8–10] c) Structure of FK-2. FK-2 was obtained by deletion of the GC base pair marked in gray in FK-1. d) Cleavage activity of FK-2 in absence and presence of 200 nM HIV-1 RT (RT*). e) Structure of FK-3. FK-3 was obtained by insertion of an additional GC base pair (marked in gray). f) Cleavage activity of FK-3 in absence and presence of 200 nM HIV-1 RT (RT*).

digest of FK-3 shows a cleavage pattern that is similar to that of FK-1 in its active form (Figure 2).

Thus, the fusion construct FK-1 represents a reporter ribozyme that can be regulated by HIV-1 RT. FK-1 reports the presence of HIV-1 RT with highest sensitivity; half-maximum inhibition of the aptazyme is already observed at a protein concentration of 10.4 nM (Figure 3a). This enhanced sensitivity reflects the extraordinarily high affinity of the aptamer-HIV-1 RT complex of 25 pM.[19] Compared to the few protein-regulated ribozymes described in the literature, this represents an improvement of sensitivity of more than two orders of magnitude. A ligase evolved by SELEX requires 1.5 μM lysozyme for half-maximum effect.[19] Micromolar concentrations of a fusion of HIV Rev and bacteriophage λN proteins are necessary for regulation of a Tetrahymena group I intron ribozyme.[20]

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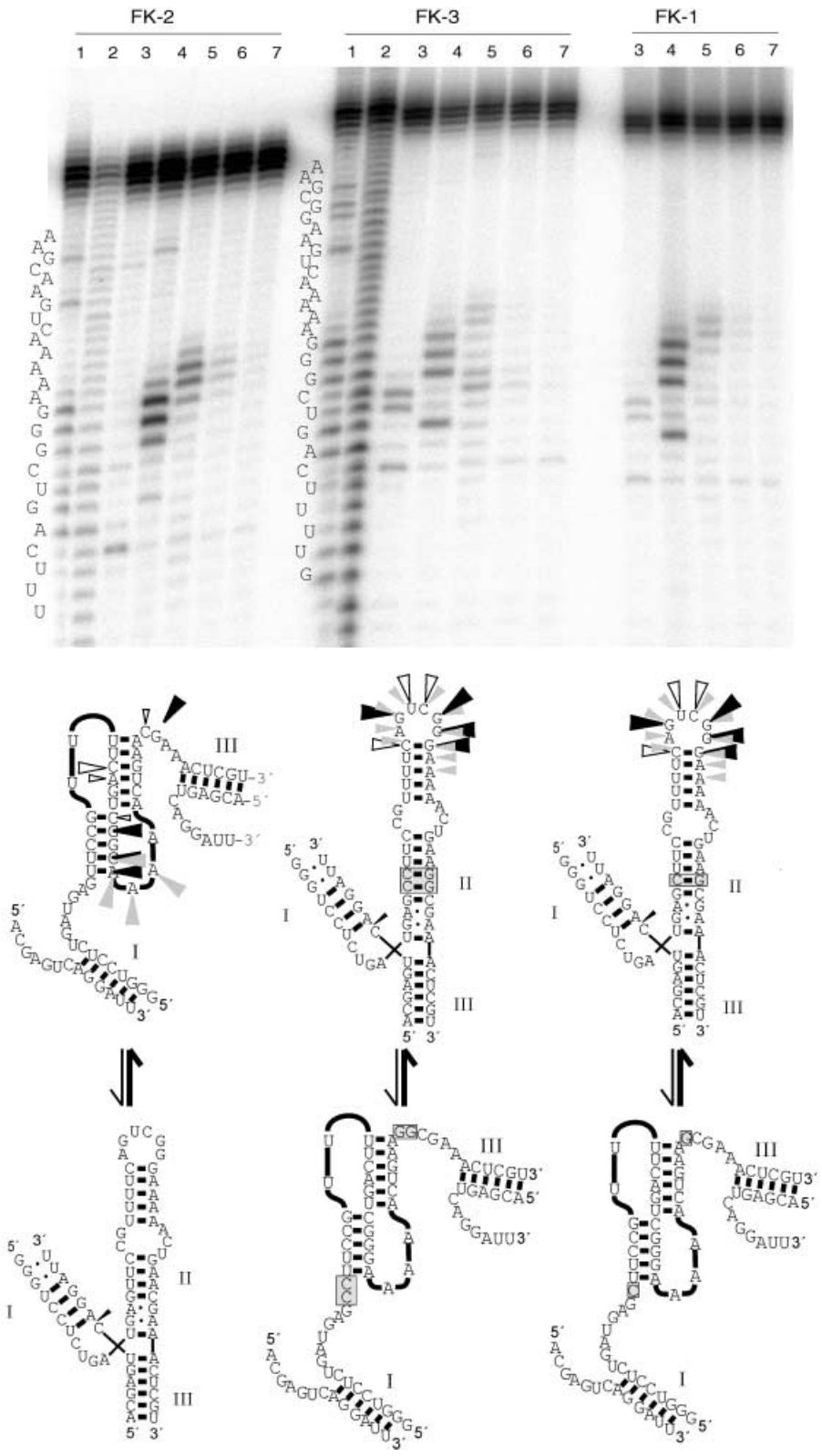

Figure 2. Nuclease-digest of fusion constructs FK-1, FK-2, and FK-3: lane 1: RNase T1 ladder under denaturing conditions; lane 2: OH ladder; lane 3: RNase A; lane 4: RNase T1; lane 5: S1 nuclease; lane 6: S1 nuclease 1/10 dilution; lane 7: S1 nuclease 1/100 dilution. RNase A cleaves single-stranded RNA at C and U (white arrows), RNase T1 single-stranded at G (black arrows), and S1 nuclease at single-stranded nucleosides (gray arrows). The size of the arrows corresponds to the intensity of the cleavage. The nuclease cleavage sites are in good accordance with the observed ribozyme activities: FK-2 forms no hammerhead structure, as for example indicated by the strong digest by S1 nuclease at the right bottom loop of the pseudoknot. FK-3 and FK-1 only show digestion at the loop of the hairpin structure of stem II, indicating the ribozyme fold.

To investigate the specificity of the reporter ribozyme, we carried out cleavage reactions in the presence of various noncognate proteins. As shown in Figure 3b, from a collection of ten proteins, only HIV-1 RT was able to significantly inhibit ribozyme activity (Figure 3a and b). Even with the homologous reverse transcriptase of HIV-2, no effect is observed. These results demonstrate that the HIV-1 RT dependent ribozyme described in this study is a highly specific reporter for HIV-1 RT. That this discrimination, which is also found for the free aptamer, is maintained in the ribozyme context demonstrates that the folded pseudoknot retains its functional properties, without the rest of the fusion construct having a disturbing influence.

To determine if the inhibition of the aptazyme FK-1 is reversible, we used the fact that the aptamer binds to the primer/template binding site of HIV-1 RT.[16] Thus the addition of a DNA primer/template complex[21] to the cleavage reaction should lead to competitive displacement of the pseudoknot-containing FK-1 from the protein, which should in turn result in a reactivation of the cleavage reaction. Figure 3c shows that this is indeed observed. The effect is even more pronounced by addition of free aptamer instead of the DNA primer/template complex (see Figure 1 in the Supporting Information). However, ribozyme activity decreases again at high concentrations due to hybridization of the free aptamer with the reporter ribozyme (see Figure 1 in the Supporting Information).

With respect to the domain-specificity of the reporter ribozyme we were interested in whether the competition effect observed with the free aptamer or the DNA primer/template complex would be specific for binders of the primer/template recognition site. This was investigated by adding nevirapine instead of the primer/template complex. Nevirapine is an inhibitor of RT binding at a region near the active site and not the primer/template recognition domain.[22] Figure 3c shows that even with higher concentrations of nevirapine no reactivation of the ribozyme is observed. This is an important result because it shows that reporter ribozymes can be used as probes in high-throughput screening formats for identification of binding molecules in a domain-specific fashion. In the example described

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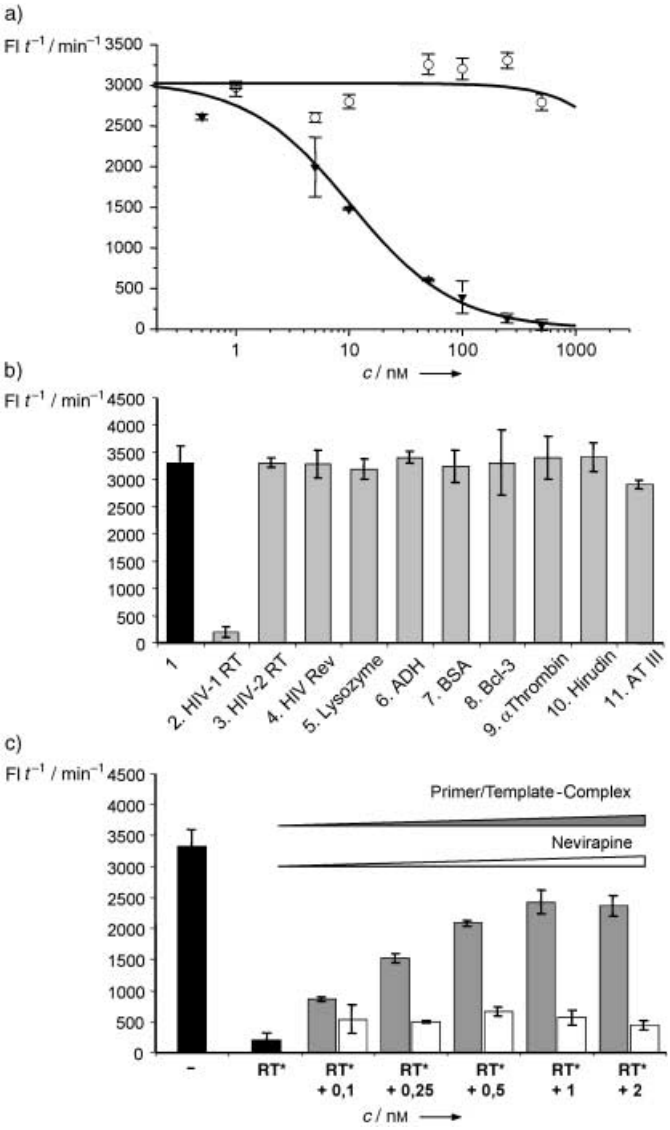

Figure 3. Concentration dependence and specificity of the inhibition of FK-1. a) Concentration dependence of the inhibition of FK-1 by HIV reverse transcriptase (filled triangles: HIV-1 RT, open circles: HIV-2 RT), half-maximum inhibition at $K_I = 10.4$ nM. b) Specificity of inhibition of FK-1. From a collection of randomly chosen control proteins, only HIV-1 RT was able to inhibit the reporter ribozyme. Protein concentration was always 200 nM. 1: without protein; 2: HIV-1 RT; 3: HIV-2 RT; 4: HIV-1 Rev; 5: Lysozym from hen egg white; 6: ADH, alcoholdehydrogenase; 7: BSA, bovine serum albumin; 8: Bcl-3, a member of the IκB-family; 9: hirudin; 10: human γ-thrombin; 11: AT III, antithrombin III. c) Competition of the aptazyme FK-1 from the HIV-1 RT by a DNA-primer/template complex. Black columns: cleavage activity of fusion construct FK-1 alone (–) and in presence of 200 nM HIV-1 RT (RT*). Gray columns: Reactivation of the FK-1-catalyzed cleavage reaction by increasing amounts of DNA–primer/template complex in presence of 200 nM HIV-1 RT (RT*). White columns: Increasing concentrations of nevirapine, a NNRTI (non-nucleoside reverse transcriptase inhibitor) that does not compete with the aptazyme for the same binding site of HIV-1 RT, is not able to reactivate the reporter ribozyme.

here, molecules are identified that bind to the primer/template binding site but not to the active site. HIV-1 RT inhibitors that interact with the primer/template binding site are not known to date.

To our knowledge, the introduced fusion construct FK-1 represents the first example of an artificially constructed switch based on a pseudoknot structure. FK-1 shows very high sensitivity and specificity. Thus, the concept of inserting pseudoknot motifs into functional RNA sequences represents a novel principle for constructing RNA switches, also applicable for natural pseudoknot/protein complexes.

Experimental Section

Synthesis of fusion constructs FK-1, 2 and 3: The corresponding DNA sequences of the fusion constructs including a 5′-T7-RNA polymerase promoter sequence were synthesized by standard phosphoramidite solid-phase technology, amplified by PCR, transcribed by T7 RNA polymerase, and purified as described in reference [23].

Ribozyme reactions: Reactions were carried out in 50 mM Tris HCl-buffer, pH 7.9, 25 mM NaCl. Reactions contained 10 nM of RNA fusion constructs FK-1, -2 or -3, 500 nM fluorescence labeled substrate,[8–10] and optional varying protein concentrations in a final volume of 50 μL. After incubation for 15 min at 37 °C, the reactions were started by adding $MgCl_2$ to a final concentration of 8 mM. The increase of fluorescence in the initial 5 min was measured as a function of catalytic activity.[8–10] Increase of fluorescence from control reactions lacking ribozyme was subtracted automatically for correction of artificial effects such as dye bleaching.

Nuclease reactions: The digestions were carried out at 37 °C in the presence of 200 μM of a noncleavable ribozyme substrate (2′-deoxycytosine at the reactive cleavage site). The reactions contained 8 μM of fusion construct and traces of 5′-^{32}P-labeled construct in 10 mM Tris buffer, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$ in the case of RNase A and RNase T1. S1 Nuclease reactions were carried out in the reaction buffer supplied by the manufacturer (Promega). After precipitation with ethanol and separation by 10 % PAGE, cleavage fragments were analyzed by autoradiography.

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