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Sequence-Specific Detection of MicroRNAs by Signal-Amplifying Ribozymes

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The sequence-specific and sensitive detection of nucleic acids is becoming increasingly important in applications ranging from basic research to diagnostics and genomics. While indirect detection based on the polymerase chain reaction (PCR), transcription-mediated amplification (TMA), or rolling circle amplification is sufficient for many of these purposes, only few methods, most commonly in situ hybridization with radiolabeled or fluorescent probes, are available for the direct detection of a nucleic acid sequence in cells, tissues, or entire organisms. The detection and quantification of small amounts of nucleic acids under these conditions often remains challenging. For example, it would be extremely useful to be able to directly detect and quantify small functional RNA sequences such as microRNAs (miRNAs) or riboswitches, that regulate gene expression,[1] as this might enable a direct profiling of their expression loci inside a cell, tissue, or organism.

Besides in situ hybridization, an interesting alternative for in situ detection of these targets would be molecular beacons as they require fewer steps in handling. These probes typically consist of a short DNA oligonucleotide, complementary to the target sequence, that forms a stem−looped structure.[2] For a direct detection of target sequences, however, molecular beacons share the same restriction as in situ hybridization probes with respect to sensitivity, due to the lack of signal amplification.[3]

Here we investigated whether the direct detection of RNAs can be achieved with ribozymes, by taking advantage of their ability to perform RNA-cleavage reactions, under multiple turnover, and their potential to be regulated by external oligonucleotides. We report a novel approach for miRNA detection based upon hairpin ribozymes that cleave a short RNA substrate labeled with a fluorophor at the 3′- and a quencher at the 5′-end, as a function of the presence or absence of a miRNA effector. This design enables real-time monitoring of ribozyme activity via FRET read-out.[4]

Catalysis of RNA cleavage by the hairpin ribozyme depends on its conformational flexibility during the docking of two helical domains A and B.[5] On the basis of this mechanism, we have designed variants of the hairpin ribozyme that can be induced by external effector oligonucleotides that interfere with the docking process. This was done by incorporating domain C, which is complementary to the target nucleic acid and also contains a short sequence that can partially pair with domain A, thus rendering the ribozyme inactive. When the complementary target sequence is added, it hybridizes with domain C and forms a pseudo-half-knot structure.[6] Domains A and B can dock again, resulting in cleavage of the substrate and generation of a fluorescence signal.[7] Figure 1 shows the verification of this concept in an inducible hairpin ribozyme, **iHP-let7**, that responds to the miRNA let-7 of *Drosophila melanogaster*.[8] The initial velocity of the cleavage reaction is shown as the increase of fluorescence per time. **iHP-let7** shows only poor catalytic activity in the absence of let7. However, upon addition of equimolar amounts of the let7, its reaction rate is induced more than 10-fold.

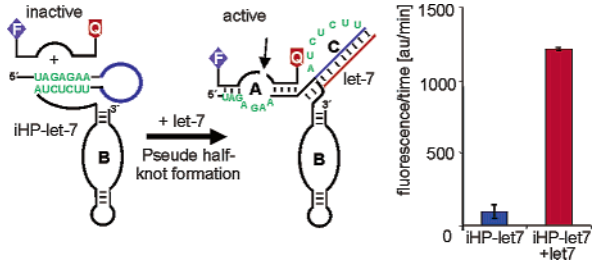

Figure 1. Reaction scheme of the hairpin ribozyme **iHP-let7** responsive to miRNA **let-7**. Domain C (blue/green) is inserted between domains A and B (middle). C contains a region complementary to the target RNA (blue) and a region that can hybridize to A (green), preventing A and B from docking and rendering **iHP-let7** inactive (left). **iHP-let7** cleaves the fluorophor (F)- and quencher (Q)-labeled substrate when **let-7** (red) binds to its complementary region in domain C (middle). (Right) Activity of **iHP-let7** in absence (blue) and presence of **let-7** (red). Reaction conditions: 50 nM **iHP-let7**, 50 nM **let-7**, reaction volume 50 μL.

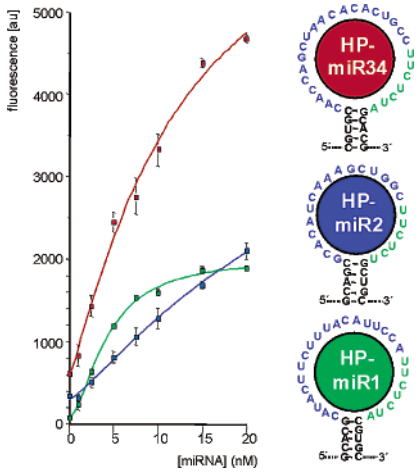

Figure 2. Three examples of domain C constructs and their concentration-dependent activity after 45 min. At this timepoint, all reactions still were in the initial, linear phase. Red: Domain C of **HP-miR34** (10 nM) and cleavage activity at increasing concentrations of **miR34**. Blue: Analogous for **miR2**. Green: Analogous for **miR1**. Sequences in blue: miRNA-hybridizing region; Sequences in red: hybridizing to domain A. Reaction conditions: 10 nM iHP, 1−20 nM miRNA, reaction volume 10 μL.

The increase of catalytic activity occurs in a concentration-dependent fashion. This was demonstrated for nine examples of ribozymes which were activated by nine different miRNAs from *Drosophila*: iHP-miR1, iHP-miR2, iHP-miR4, iHP-miR5, iHP-miR7, iHP-miR10, iHP-miR34, iHP-miR79, and iHP-let7. Figure 2 shows three representative examples: miR34, miR2, and miR1.

All ribozymes exhibited very low cleavage activity in the absence of the corresponding miRNA and were activated when it was added. Significant increase in fluorescence was already detectable at miRNA concentrations as low as 5 nM (Figure 2), corresponding to a detection limit of 50 fmol miRNA in the reaction mixture.

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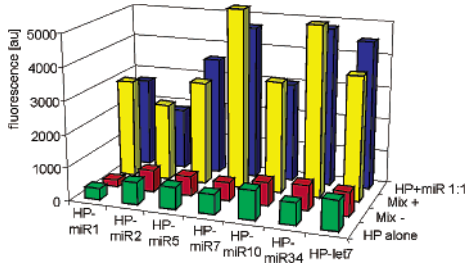

Figure 3. Activity of hairpin ribozymes (10 nM) in absence of miRNA (green), in the presence of a mixture of miRNAs without the corresponding one (red), in the presence of a mixture of all miRNAs (yellow), and in the presence of the complementary miRNA only (blue). Reaction conditions: 10 nM iHP, 10 nM miRNA, reaction volume 10 μL.

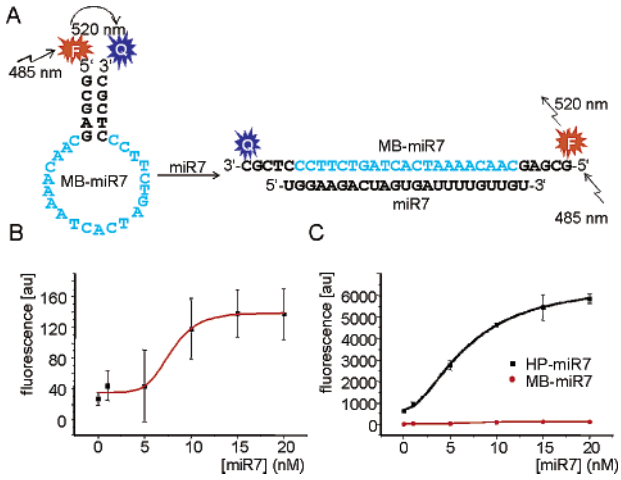

Figure 4. Comparison of **MB-miR7** with **HP-miR7**. (A) Sequence of **MB-miR7**. red: complementary sequence to **miR7**. F: 2-Carboxyfluorescein, Q: Dabcyl. (B) Fluorescence of **MB-miR7** (10 nM) at 1−20 nM **miR7**. (C) Comparison of the fluorescence of **MB-miR7** with **HP-miR7** (50 mM Tris pH 7.5, 30 mM $MgCl_2$, 10 nM MB/HP, **miR7**. Volume: 10 μL).

Next, we analyzed the specificity of the inducible ribozymes. For this purpose, ribozyme reactions for the nine different constructs were performed in a reaction mix that contained the whole series of miRNAs in equimolar amounts (Figure 3, lane "Mix+"). The fluorescence signals obtained approximately matched the values derived from reactions with only the cognate sequence present (lane "HP+miR 1:1"). As negative controls, we measured the activity of each construct without any miRNA (lane "HP alone), or in the presence of a mix of equimolar amounts of all other eight miRNAs except the corresponding one (lane "Mix-"). For all nine constructs the obtained fluorescence was comparable to the values derived from reactions lacking any miRNA. This result impressively shows that the activation of the ribozymes by miRNAs is highly sequence-specific with no detectable cross-reactivity to noncognate sequences.

Having established the sensitive and highly sequence-specific detection of miRNAs with the **iHP-miR** system we were interested in how the ribozymes compare with standard molecular beacons for nucleic acid sequence detection. For this purpose, a molecular beacon for miRNA 7 was constructed (**MB-miR7**). It consists of a DNA sequence complementary to microRNA 7 which is flanked by five additional nucleotides on both ends for stem formation (Figure 4A).[9] Both **MB-miR7** and **HP-miR7** reactions were done under exactly the same conditions in a 10 μL volume to directly compare their sensitivities. Incubation of **MB-miR7** with increasing concentrations of miRNA 7 resulted in increasing fluorescence (Figure 4B). Although the molecular beacon is opened in a concentration-dependent fashion, the obtained standard deviation is relatively high due to a low signal-to-noise ratio obtained at these low concentrations of molecular beacon and target RNA. For better comparison, the results generated with the **HP-miR7** and **MB-miR7** are shown in the same scale in Figure 4C. The ribozyme yielded at least 20-fold higher fluorescence, presumably due to multiple turnover of substrate cleavage. At higher concentrations of the molecular beacon and target RNA, higher fluorescent signals with less deviation were obtained (Supporting Information, 3). Thus, the molecular beacon used in this study functions normally but detects the target sequence with a sensitivity of at least an order of magnitude lower than that for the ribozyme approach.

In conclusion, we show that hairpin ribozymes can be designed in a rational and straightforward way, that can be induced by external oligonucleotides sequence-specifically. Due to intrinsic signal amplification (Supporting Information 1), their sensitivity is at least an order of magnitude increased compared to that of standard molecular beacons. These probes may be useful in applications that require direct detection of nucleic acids within their natural environment. For applications in vivo or within tissues, however, it will be necessary to increase the stability of **iHP**-derivatives and the substrate against ribonuclease digestion. We are currently exploring the possibility to utilize stabilized variants of the introduced ribozymes for nucleic acid sequence detection in situ or in vivo.

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Supporting Information Available: Experimental details on synthesis and sequences of oligonucleotides, ribozyme and beacon reactions, DNA sequences (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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