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Real-Time Characterization of Ribozymes by Fluorecence Resonance Energy Transfer (FRET)**

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Ribozymes are increasingly applied to the inhibition of gene expression at the level of protein-encoding mRNAs.[1, 2] The possibility of transferring ribozymes endo- or exogenously into living cells opens up a broad potential for application in gene therapy,[1, 3-5] functional genomics,[6] and biotechnology.[7] The ribozyme most commonly used for these purposes is the hammerhead ribozyme (HHR), a small catalytic RNA that is able to cleave other RNA molecules in an intermolecular fashion.[8] The specificity of this cleavage process is determined by substrate binding sites that are variable in sequence and length. As a result, the cleavage activity can be directed against almost any mRNA sequence. Despite their potential for universal application there are a number of factors that have to be taken into account when developing therapeutic ribozymes, for example, the accessibility of ribozyme binding sites on the target RNA in vivo,[9] the selectivity of substrate recognition,[1] or the cleavage efficiency in an intracellular compartment.[10, 11] Although computer-assisted predictions can be made to address some of these points[12] they still have to be verified experimentally by characterizing the respective HHR-constructs under various conditions. Therefore, there is currently a high demand for technologies that allow a high-throughput screening of ribozymes for a rapid qualitative evaluation of their kinetic parameters, especially as current conventional methods hardly provide sufficient solutions to this problem.

Here we report a novel approach based on FRET-oligonucleotides[13] (FRET = fluorescence resonance energy transfer[14]), which allows the real-time kinetic analysis of ribozymes within hours. The FRET-principle has previously been applied to various problems such as determination of phosphodiesterase activities,[15] structural elucidations of RNA molecules,[16, 17] and for quantification of PCR reactions.[18] Intermolecular FRET-measurements with single-labeled substrates and hammerhead ribozymes have also been applied to measure the association and dissociation of substrate and product.[19, 20]

In our FRET-substrate the fluorescence of a fluorophore (for example, 6-carboxyfluorescein (FAM)) is intramolecularly quenched because of the close spatial proximity of a fluorescence-quenching molecule (for example, 6-carboxytetramethylrhodamine (TAMRA); Figure 1a, b). Upon cleavage of such substrates by the ribozyme a fluorescence-signal is generated which can be quantified by an appropriate read-out system in real time (Figure 1c). As the increase in fluorescence is directly correlated with the rate of cleavage, this system is well suited for the sensitive, nonradioactive, rapid analysis of ribozyme activities.

Based on a published HHR/substrate complex (Figure 1a) we have constructed a FRET substrate (SL1), which was used for real-time determination of the activity of the ribozyme HHR1. An inactive HHR-mutant ($HHR1_{mut}$) with substrate binding sites identical to those in HHR1 was used as a reference and negative control. The Michaelis–Menten parameters of HHR1 could be obtained in a single experimental setup by measuring the increase in fluorescence as a result of cleavage at different substrate concentrations. The result of this experiment is shown in Figure 2b in the form of an Eadie–Hofstee plot for the determination of the k_{cat}/K_M values. To investigate whether the FAM/TAMRA label affects the cleavage efficiency we determined the kinetic parameters by conventional methods[22] with a ^{32}P-labeled

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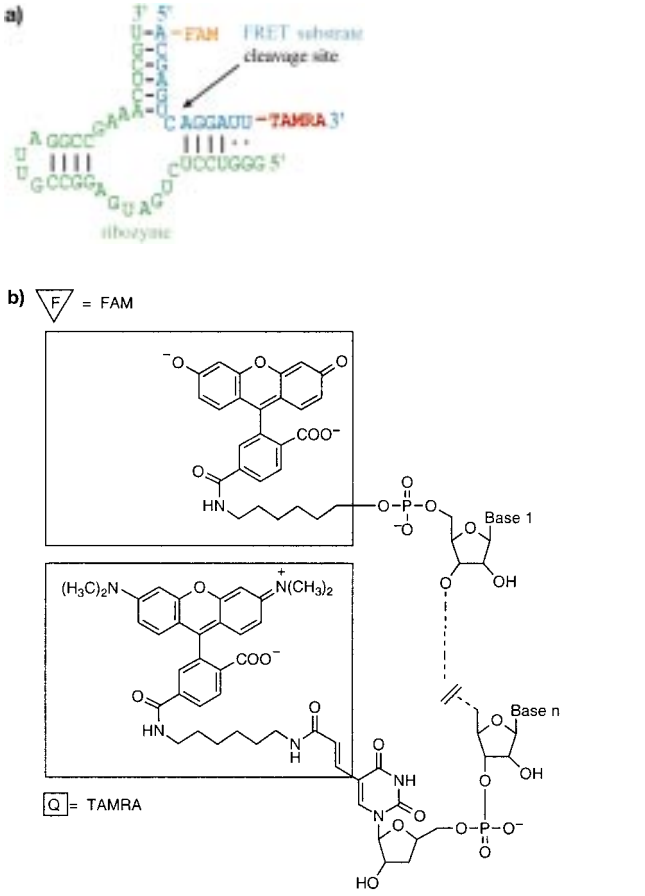

Figure 1. Schematic representation of the assay and automated analysis. a) Secondary structure of ribozyme and substrate. b) The fluorophore (FAM) and quencher molecule (TAMARA) c) Up to 96 different reactions can be monitored on a microtiter dish in real time and the data analyzed with an appropriate read-out system. The reaction is shown schematically on the left. 1) Binding of the doubly labeled RNA substrate (e.g. 5′-[F] and 3′-TAMRA [Q]-labeled RNA) to the ribozyme results in the formation of the catalytic complex. The fluorescence of the donor ($\lambda = 535$ nm) is intramolecularly quenched in the uncleaved substrate. 2) Cleavage of the doubly labelled substrate. 3) Dissociation of the reaction products. The ribozyme is now available for the next turnover. Neutralization of the quench-effect results in the increase of a fluorescence signal $h\nu'$ as a function of the cleavage of the substrate by the ribozyme.

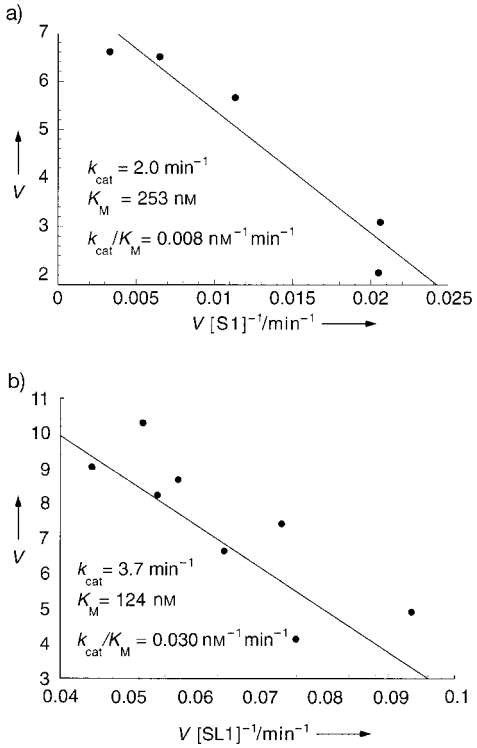

Figure 2. Determination of the kinetic parameters of HHR1 ($c = 4$ nM) at 37 °C. Eadie-Hofstee plot and kinetic parameters of cleavage of 5′-^{32}P-labeled substrate, which carries no FRET labels S1, by HHR1 a) and b) of the FRET-substrate (SL1) by HHR1.

substrate (S1) without the FRET labels (Figure 2a). The conventional method involves separation of the cleavage products by electrophoresis on polyacrylamide gel with subsequent quantification of the bands by autoradiography.[22] Figure 2 shows the data for both substrates (S1 and SL1). Both the k_{cat} and the K_M values are of the same order of magnitude, which indicates that the FRET substrate is very well suited for our objective.

To test whether the method can also be applied to the rapid characterization of ribozymes we measured cleavage activities in parallel experiments in which various physicochemical parameters were altered. Figure 3a shows the time-course of the cleavage reaction of an experiment under standard conditions. In Figures 3b–d the reaction rate is shown as a function of pH, temperature, and Mg^{2+} concentration. Under the conditions applied here, we obtained an optimum pH value of 8 and an optimal Mg^{2+} concentration of 8 mM. The optimum temperature of 30 °C is rather low and probably reflects the relatively weak binding of the substrate to the ribozyme. This might be a consequence of the short hybridizing arms and the low Mg^{2+} concentration (4.0 mM).[23] Finally, we determined the effect of the aminoglycoside neomycin B, a HHR inhibitor, on substrate cleavage (data not shown). The cleavage activity dropped to a third of the noninhibited reaction rate in the presence of 100 μM neomycin, which is in exact accordance with the data obtained by conventional methods in a previous study.[24]

The data points were corrected with the ribozyme-independent negative-control values obtained with the inactive

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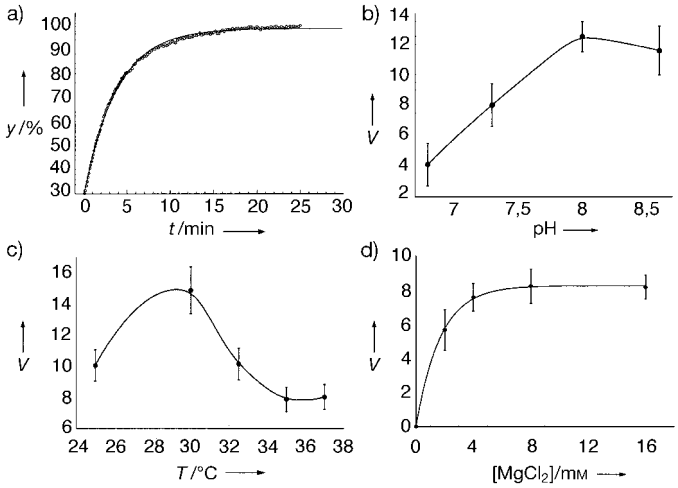

Figure 3. Characterization of the cleavage of 200 nM and 300 nM SL1 with 4 nM HHR1. a) Time course of cleavage with the reciprocal of the exponential regression curve (only 1% of all data points are shown). $y =$ amount of cleavage. b – d) Dependence of the reaction rate V of HHR1 on b) the pH value, c) temperature, d) the concentration of Mg^{2+}. The values of k_{cat}/K_M at 25 °C (0.0134 $nM^{-1}min^{-1}$) and 30 °C (0.138 $nM^{-1}min^{-1}$) were also determined.

mutant $HHR1_{mut}$ to eliminate possible intrinsic instrumental inaccuracies or unspecific fluorescence effects (for example, fluctuation of the signal as a result of deviations in temperature). In addition to that, this method of adjustment enabled the determination of the activity of ribozymes generated in situ by T7 RNA polymerase dependent transcription of ribozyme-encoding DNA-templates in the presence of HeLa-nuclear extracts (Figure 4). As a control we carried out

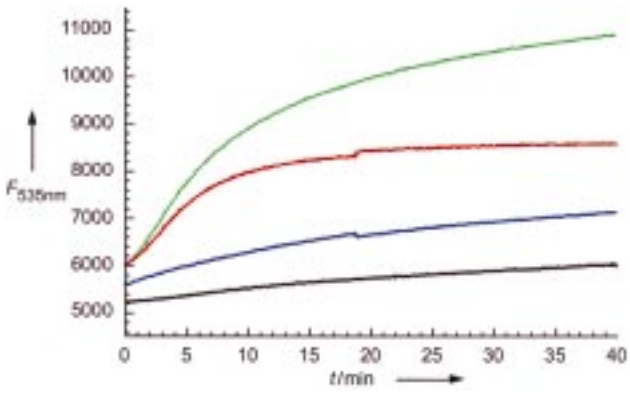

Figure 4. Real-time monitoring of the increase of fluorescence as a result of the activity of HHR1 generated in situ in the presence of nuclear cell extract. $HHR1_{mut}$-DNA + T7 RNA polymerase (black), HHR1-DNA without T7-RNA polymerase (blue), HHR1-DNA + T7-RNA polymerase (green). The red curve refers to the same measurement but corrected for the unspecific background signal (see experimental section).

parallel experiments in the absence of T7 RNA polymerase (blue) and with the incactive HHR-mutant (black). Both curves show a slight increase in the fluorescence signal, which might result from the unspecific degradation of the FRET substrate by RNases in the nuclear extract. The green curve shows the absolute fluorescence that was obtained with HHR1 generated in situ. The same data, corrected for the superimposed background reaction (see experimental section), are represented by the red curve. As expected, the corrected curve reveals a sigmoidal shape that reflects the continuous increase of ribozyme concentration during transcription. These results show that it should in principle be possible to use the ribozyme as a reporter for measuring transcription rates in vitro and in vivo. Such a reporter system could be part of a high-throughput screening assay to search combinatorial compound libraries for inhibitors of transcription factors since it can be used fully automated without the need of additional pipetting steps.

In conclusion, we have shown that doubly labeled FRET substrates allow the characterization of ribozymes under various conditions in only a fraction of the time estimated for conventional methods currently available. In particular the very simple automatization and real-time analysis of many parallel reactions and the avoidance of radioactive labeling perfectly suits our system for the high-throughput screening of ribozyme activities and transcription rates.

Experimental Section

HHR1: the sense-DNA-strand of the ribozyme with the 5′-terminal T7-promotor sequence was synthesized on an Expedite oligonucleotide synthesizer (Millipore, USA): 5′-*TCTAATACGACTCACTA*-*TA* GGG TCC TCT GAT GAG *GCC GTT AGG CCG AAA CTC GT*-3′ (HHR1-DNA; the primer binding sites are shown in italics). The synthesis of the antisense strand as well as the amplification of the double-stranded template was done by polymerase chain reaction (PCR) with primers 5′-TCTAATACGACTCACTATA-3′ (5′-primer) and 3′-GGCAATCC-GGCTTTGAGCA-5′ (3′-primer). A standard 100 µL PCR reaction contained 10 mM Tris-HCl, pH 8.9, 100 mM KCl, 1.5 mM $MgCl_2$, 50 mg mL^{-1} BSA, 0.05% Tween 20 (v/v), 200 µM dNTP, 2 µM 5′- and 3′-primer, approximately 200 nM single-stranded DNA-template, and 2.5 U Tth DNA polymerase. Amplification was performed according to the manufacturers protocol (Roche Mannheim) in four to five PCR cycles (95 °C, 55 s; 55 °C, 1 min; 72 °C, 1 min). Amplified DNA was isolated by standard methods[25] and excess primers were removed by agarose gel electrophoresis.

The inactive ribozyme mutant HHR1mut was generated accordingly using the synthetic DNA-template 5′-*TCT AAT ACG ACT CAC TAT A* GGG TCC TCT **TAG** GAG *GCC GTT AGG CC**A** **G**AA CTC GT-3′* (HHR1mut; primer binding sites are shown in italic, four mutations are shown in bold) and the primers 5′-*TCT AAT ACG ACT CAC TAT A*-3′ (5′-primer), 3′-*GG CAA TCC GGT CTT GAG CA*-5′ (3′-primer). The 5′-FAM- and 3′-TAMRA-labeled substrate RNA was synthesized and purified by HPLC and gel electrophoresis at Eurogentec (Belgium). Conclusive experiments were found to strongly depended on the purity of the FRET substrate. The ratio of fluorophore and quencher dye, which is a measure of the purity of the sample, was calculated as a function of fluorescence as described.[13, 26]

A standard 50 µL reaction for the generation of ribozyme by in vitro transcription contained 40 mM Tris-HCl, pH 8,0, 50 mM NaCl, 2 mM spermidine, 5 mM dithiothreitol, 8 mM $MgCl_2$, 0.2 – 2 µM HHR1-DNA or HHR1mut-DNA, respectively, 4 mM A/C/G/UTP, 40 U RNAsin (Promega, Madison, WI), and 50 U T7 RNA polymerase (Stratagene, Heidelberg). The RNA was purified on a 16% denaturing polyacrylamide gel (37.5:1). The sample also contained SL1 and 10 U HeLa-nuclear extract (HeLa Cell Extract Transcription System, Promega, Madison, WI) for measuring ribozyme activities of HHR1 generated in situ. In vitro transcription was started by the addition of polymerase to the nuclear extract and incubated at 37 °C during the course of one measurement.

Real-time measurements of the ribozyme dependent increase of fluorescence were done in 50 µL volumes. The signal obtained reflects the chemical-cleavage step in addition to the dissociation of the cleavage products. Fluorescence quantifications in the transcription-controlled reactions were done in an ABI Prism 770 spectrometer (Applied Bio-

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systems, USA). The emmission spectra of the two dyes were recorded at intervals of 0.5 – 0.7 s (FAM: $\lambda_{max} = 535$ nm, TAMRA: $\lambda_{max} = 582$ nm, excitation wavelength: 488 nm). All experiments were done in triplicate. By plotting the relative fluorescence of the fluorophore (ΔR_n value) at 535 nm against time, the proportion of cleaved RNA substrate versus negative control (identical experiment with inactive HHR1mut-DNA instead of HHR1-DNA) was determined. The raw data were imported into Microsoft Excel, computed, and further processed.

To eliminate unspecific fluorescence effects it had to be taken into account that the absolute, but not the percental, deviation in fluorescence increases with the increase in fluorescence during the catalytic cleavage. The time course of the cleavage was corrected by the percental deviation of the curve obtained for the negative control experiments from the value at $t = 0$ according to Equation (1) ($F_{korr}(t)$: corrected fluorescence at a given time point t; $F_{neg}(t)$: fluorescence of the negative control at a given time point t; $F_{neg}(t_0)$: fluorescence of the negative control at $t = 0$; $F_{HHR}(t)$: increase in fluorescence in the presence of transcribed HHR at a given time point t).

$$F_{korr}(t) = \frac{F_{neg}(t)}{F_{neg}(t_0)} F_{HHR}(t) \quad (1)$$

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Exploiting Incommensurate Symmetry Numbers: Rational Design and Assembly of $M_2M'_3L_6$ Supramolecular Clusters with C_{3h} Symmetry**

Xiankai Sun, Darren W. Johnson, Dana L. Caulder, Ryan E. Powers, Kenneth N. Raymond,* and Edward H. Wong*

There are many elegant oligomeric or polymeric structures based on metal – ligand interactions that lead to well-organized supramolecular architectures. Examples include extended arrays[1–7] in two or three dimensions and discrete clusters of various types.[8–30] However, relatively few such clusters have been the result of predictive design. We have developed a rational design for the synthesis of high-symmetry clusters using coordination number incommensurate interactions. Resultant examples include M_2L_3 helicates[15, 24, 31, 32] and their *meso*-counterparts (mesocates),[32] and M_4L_6[12, 33–35] and M_4L_4[36] tetrahedra. In these clusters, three bidentate chelators coordinate a tri- or tetravalent metal ion in a pseudo-octahedral fashion to generate a threefold axis, and a symmetric, multi(bidentate) ligand generates the other symmetry element (twofold,[15, 24, 31, 32] threefold,[36] or mirror plane[32]).

Here we present the rational design of a mixed-metal cluster in which, rather than deriving one symmetry element

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