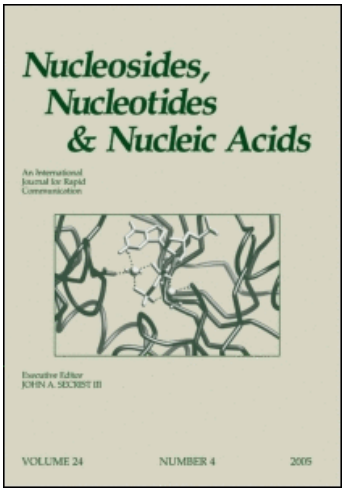

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Fluorescence Resonance Energy Transfer (Fret) to Follow Ribozyme Reactions In Real Time

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FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) TO FOLLOW RIBOZYME REACTIONS IN REAL TIME

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Abstract. Fluorescence resonance transfer (FRET) was applied for real time monitoring of ribozyme reactions. Group I ribozyme ligation was followed with two separate, fluorescent-labeled RNA substrates. For hammerhead ribozyme cleavage, a double-fluorescent-labeled substrate was used. For the first time we analyzed multiple turnover conditions. Real time monitoring permits convenient analysis of ribozyme kinetics and the sequence-specific, quantitative detection of RNAs in femtomole amounts.

INTRODUCTION

Ribozymes catalyze a wide range of RNA processing reactions, including cleavage, splicing and ligation. In conventional studies, radiolabeled substrates and products are separated by gel electrophoresis, followed by autoradiography. Kinetic studies require the analysis of a series of individual aliquots, one for each time point.

In contrast, real time monitoring yields a series of time points with any desired resolution (intervals of 60 seconds or less). No aliquots have to be withdrawn, no gel electrophoresis is neccessary. The sample is unaffected by the analysis procedure. Finally, if desired, the reaction products can be characterized by conventional gel electrophoresis and subsequent fluorescence scanning.

FRET has been shown to be a powerful analytical tool in "real time quantitative PCR" [1]. Recently, this technology was also applied to follow RNA reactions, but only single labeled RNAs were used. In this format, only single turnover reactions with ribozyme excess could be monitored. Hybridization of a fluorescein 3'-labeled substrate with a 5'-TAMRA-labeled hammerhead ribozyme resulted in proximity of both dyes. Because almost all substrate was present in the complex, fluorescence was quenched. Upon cleavage, the short products were released concomittant with release of quenching and increasing fluorescence signal [2]. In a second system, the fluorescein 3'-labeled

substrate formed a hybrid with a hairpin ribozyme. In this construct, fluorescein approached an unpaired guanosine at the juxtapposed end of the newly formed RNA helix. This association could be followed because the dangling guanosine acts as quencher. This quenching effect was shown to be specific for guanosine base and it was applied to follow cleavage of a substrate RNA in the presence of ribozyme excess [3].

For the first time, we monitored ribozyme reactions with substrate excess under multiple turnover conditions. This required a reaction dependant change of fluorescence due to conversion of substrate into product. We realized this approach for two different ribozyme systems: RNA ligation by a group I ribozyme and RNA cleavage by a hammerhead ribozyme.

RESULTS AND DISCUSSION

Our analysis required the use of two different fluorochromes, which have to be monitored independantly and quantitatively. This requirement is met conveniently by the recently available Sequence Detection System (ABI Prism 7700) with its thermocycler unit in microtiter format and its on-line fluorescence measurement through fiber optics and transparent tube covers. The software can separate the signals for the standard FRET pair for this instrument: fluoresecein, 5-FAM, as reporter dye (emission maximum 520 nm) and the rhodamine dye, TAMRA, as quencher (emission maximum 580 nm). In addition we used the cyanine dye, Cy-5, as quencher (emission maximum 670 nm) with its easily separated fluorescence signal.

In general, all anticipated results from FRET data were confirmed by subsequent gel electrophoresis, followed by detection either of ^{32}P-labeled RNAs (phosphorimager) and/or of Cy-5-labeled RNA (fluorimager).

RNA ligation by a group I ribozyme

We applied the recently published system by Doudna et al. [4], using the unimolecular ribozyme RNA. Substrate RNAs and reaction scheme are shown in FIG.1. We established that fluorescent dyes had only moderate inhibitory effects on reaction rates, with essentially no effect at position I (Cy-5 or TAMRA), ca. 60% inhibition at position II (5-FAM) and ca. 30% inhibition at position III (5-FAM).

First, we intended to apply the formation of the new RNA stem in the ligation product, resulting in proximity of positions I and III. We were surprised to find that the 6 base pair helix was sufficiently stable to promote association of both substrate RNAs, which resulted in quenching of the FAM signal, in the absence of any ribozyme reaction

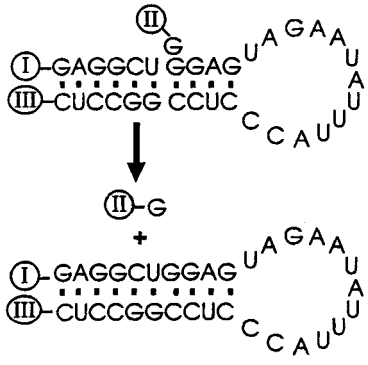

FIG.1. Structure of substrate RNAs and the ribozyme catalyzed ligation products[4]. Fluorescent labels at positions I, II, III were analyzed for interference with the ribozyme reaction.

(FIG.2A, 2B). As anticipated, the hybridization (quenching) could be disrupted by heating of the substrate RNAs, but quenching was maintained in the ligated product (FIG.2C). This would allow us to apply FRET for end-point determinations, but not for the intended real time monitoring.

We decided to apply the formation of substrate hybrids to our advantage: Both substrate RNAs were labeled, the quencher at the 5'-end of the six-mer RNA, and the reporter FAM at the 5'-terminal uridine of the larger, 30-mer RNA. We had to use an extended leaving group because FAM would be quenched by an immediately neighbouring guanosine [1,3]. In this arrangement, no quenching was observed with TAMRA at position I (data not shown), whereas substantial quenching was observed with Cy-5 (FIG.3, 4A). Quenching was not complete, possibly due to the long distance with a rigid 6 base pair stem separating the FRET pair. Ligation reaction results also in cleavage and liberation of the short leaving group (FIG.3), and the anticipated release of quenching could be observed in a time dependant manner (FIG.4A, B, C). This format is also suitable for the comparison of different reaction conditions, like the presence of the detergent SDS or urea (FIG.4C). Compounds like guanidinium isothiocyanate can also severely influence fluorescence and obscure FRET detection (FIG.4C), although they may have only minor effects on the ribozyme reaction. This was revealed by gel electrophoresis as additional monitoring with the conveniently detectable dye Cy-5 (FIG.4C, 5).

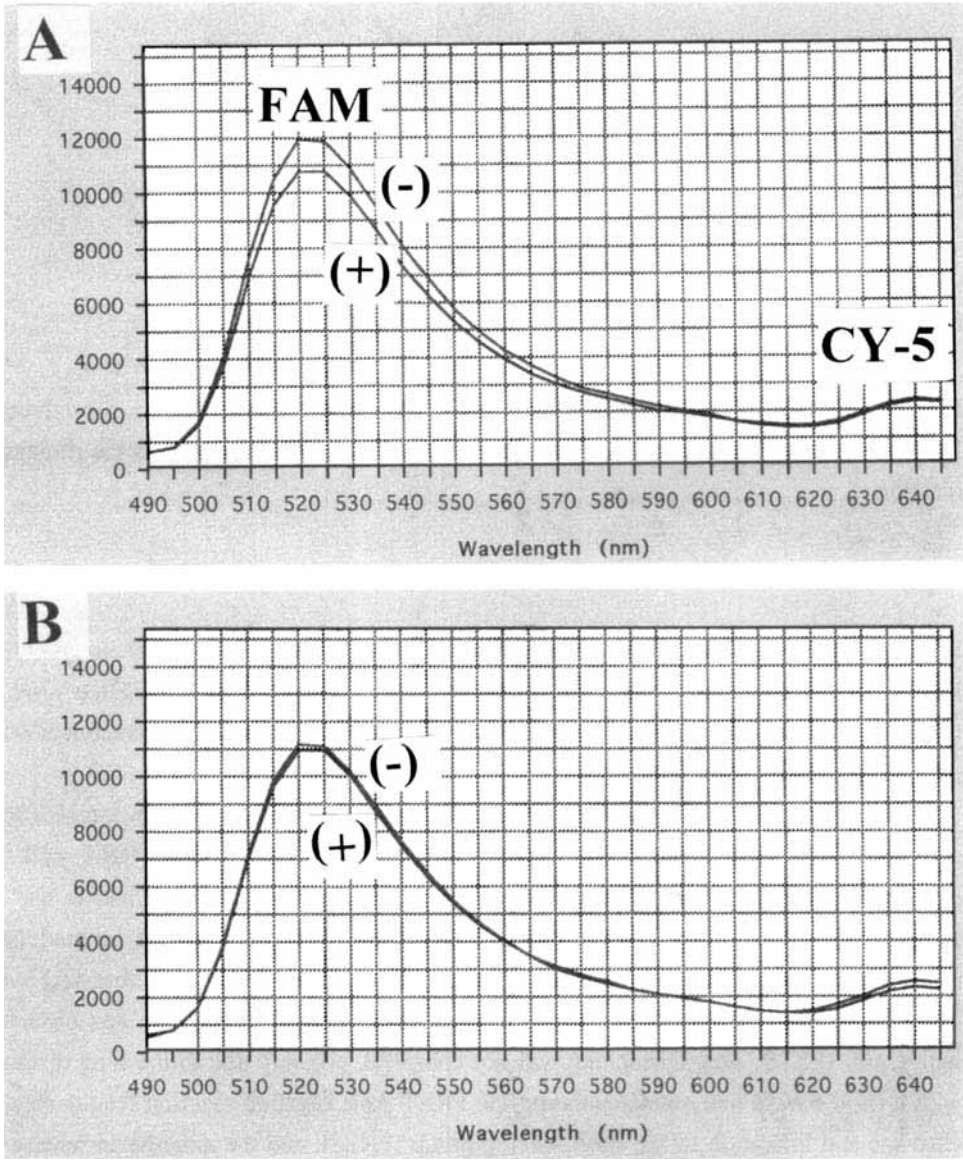

FIG.2. Fluorescence spectra of mixed substrate RNAs (2 μM each) labeled with Cy-5 (position I) and FAM (position III). Incubation at 42°C was performed for 200 min in the absence (-) or presence (+) of 2 μM ribozyme. Spectra were recorded with ABI Prism 7700 for 15 sec at 5 min intervals. Panel A: Spectra at the beginning. Cy-5 quencher signal was at the extreme right margin. Panel B: Spectra at the end of the incubation period. Panel C: Samples were heated to 65°C and spectra were recorded.

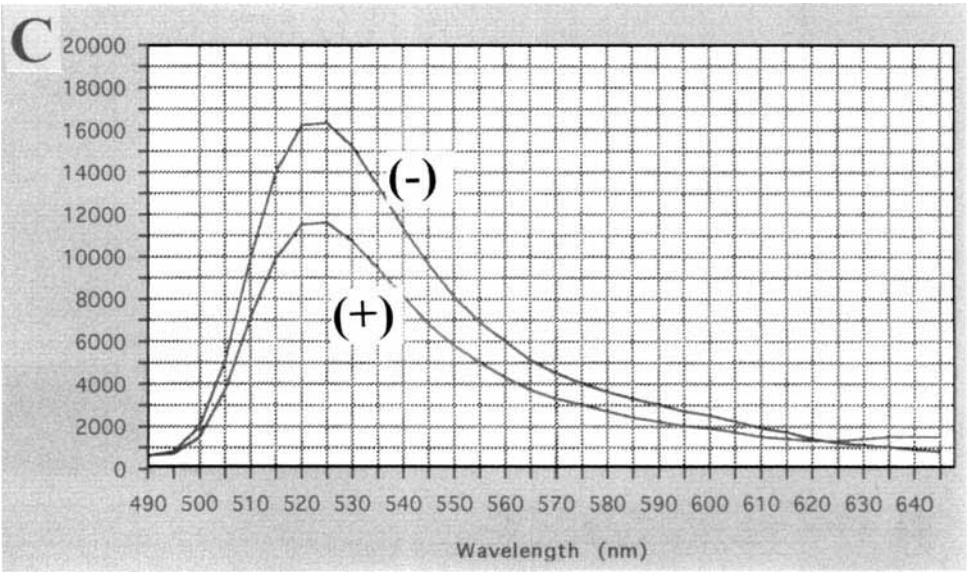

FIG. 2C

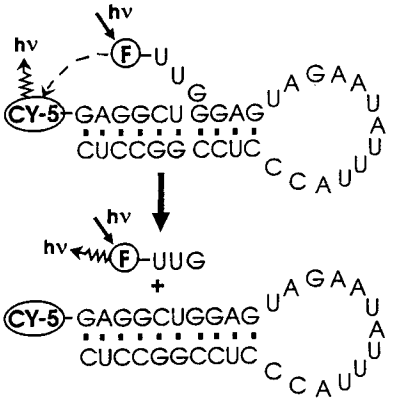

FIG.3. Finally, the preferred format for monitoring the ligation reaction. Initial FRET with energy transfer from FAM (**F**) to Cy-5 is disrupted upon liberation of the UUG leaving group.

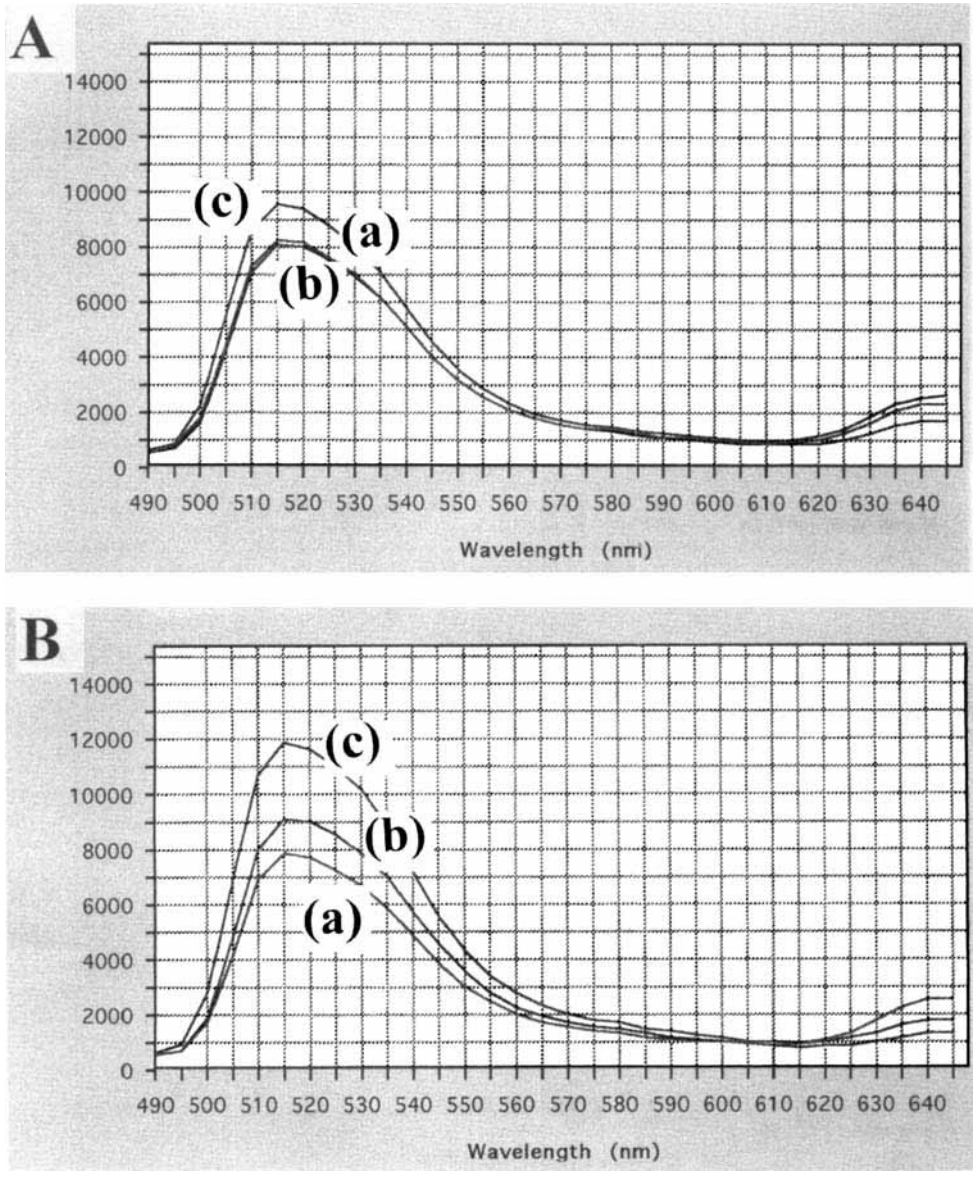

FIG.4. Fluorescence spectra of mixed substrate RNAs, as shown in FIG.2. Sample a: 2 μM of each substrate; sample b: 2 μM of each substrate and 2 μM ribozyme; sample c: 2 μM ribozyme, 2 μM FAM-labeled RNA and 5 μM Cy-5-labeled substrate. Panel A: Spectra at the beginning. Panel B: Spectra at the end. Panel C: Recording of relative FAM fluorescence versus time. Conditions were as for sample c without ribozyme (Control), with ribozyme and without further additions (None), with 100 mM urea , with 5% SDS and with 100 mM guanidine isothiocyanate (GITC).

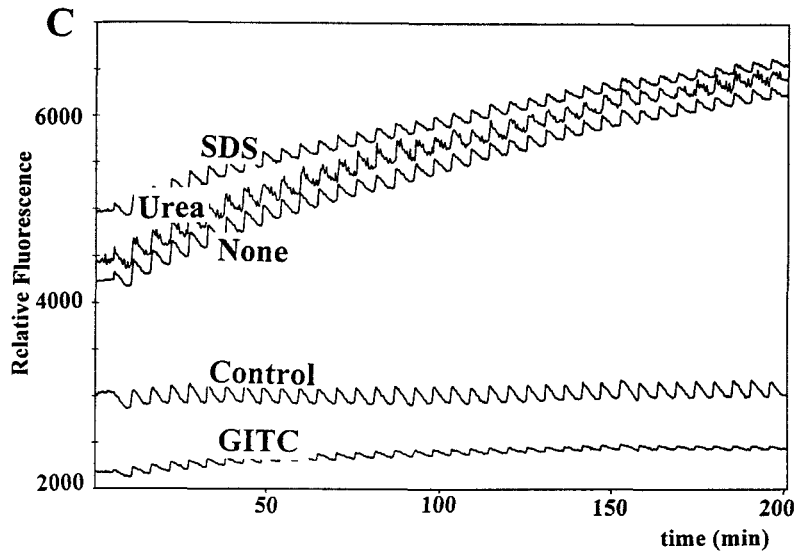

FIG. 4C

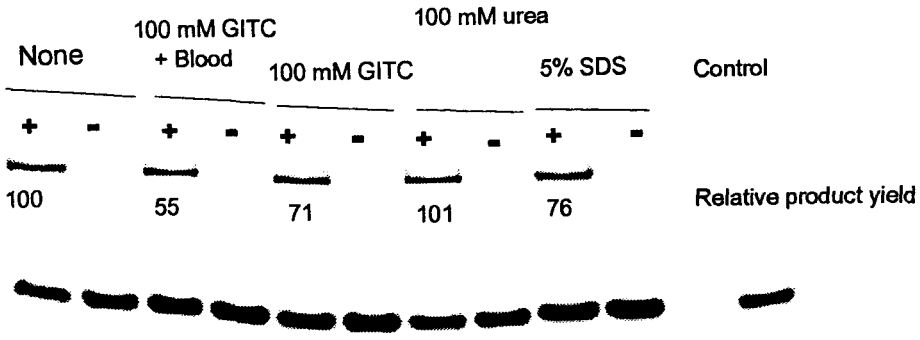

FIG.5. Samples from FIG.4C were analyzed by gel electrophoresis and Cy-5-labeled RNAs were detected with STORM imager (red mode). Additives are indicated and ribozyme was present (+) or absent (-). Relative ligation product yields are indicated, the lane without additives (None) was set as 100.

RNA cleavage by a hammerhead ribozyme

We applied the recently published system by Clouet-D'Orval & Uhlenbeck [5]. Initially we used 5'-TAMRA-labeled RNA, combined with 3'-FAM-labeling and an extra uridine to prevent proximity of FAM and the quenching guanosine (FIG.6). However, no quenching was observed with various Mg concentrations (FIG.7). This is reminiscent of the absence of quenching with TAMRA in position I (see structure in FIG.1). We conclude, that also the quencher dye should not be placed next to guanosine.

In consequence, we inserted a thymidine base with the quencher directly attached at the base moiety. Efficient quenching ensued (FIG.8A). However, in the absence of any additive, a time dependant loss of the quencher signal was observed (FIG.8B) - which required the addition of at least 1 mM Mg (data not shown). We assumed that a rearrangement occurred with concommitant disruption of the FRET pair (FIG.9A). Since both structures contain the same RNA stem, the dye seems to stabilize its neighbouring stem - by sequestering in a hydrophobic RNA pocket? Potentially, this could be avoided or at least reduced, by making the solvent more hydrophobic and more suitable to accomodate the organic dye moiety. We tried 10% DMSO, 10% EtOH, 1% SDS and 1% Triton X-100; all were effective in stabilizing the TAMRA signal (not shown), and we decided to use 10% EtOH.

Finally, we were able to monitor the hammerhead cleavage reaction (FIG.9B). In the presence of 10% EtOH and 20 mM Mg (at pH 7.5) we incubated 500 nM substrate (25 pmoles) and cleavage could be followed with decreasing amounts of ribozyme in the range of 2.7 pmoles to 100 fmoles (FIG.10A, B). The corresponding yields of cleavage after 40 min were determined by gel electrophoresis and they ranged between 80% and 5% (not shown). This means, we observed multiple turnover and one substrate RNA per min was cleaved per ribozyme.

Drastically enhanced reactions were observed at pH 8.5 and with 400 mM Mg, where comparable cleavages could be observed in the range of 90 to 3 fmoles (FIG.11A, B) with 80% to 40% cleavage after 200 min, as determined by subsequent gel electrophoresis (not shown). This means, about 17 substrate RNAs per min were cleaved per ribozyme. In addition, FRET is displayed by the fluorescence ratio (reporter signal divided by the quencher signal) versus time (FIG.12). Finally, ribozyme concentration was plotted against the time elapsed until the value of 1.0 was reached for the fluorescence ratio of the corresponding ribozyme concentration (FIG.13).

CONCLUSIONS

(i) Avoid reporter and quencher dyes next to guanosine base.

(ii) Fluorescent-labeled RNAs retain (most of) their activity as ribozyme substrates.

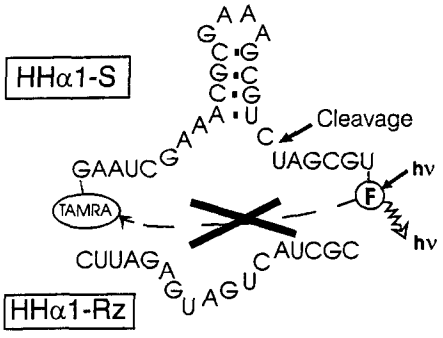

FIG.6. Structures of substrate (HHα1-S) and hammerhead ribozyme (HHα1-Rz). Cleavage position is indicated. The anticipated FRET was not observed (see FIG.7).

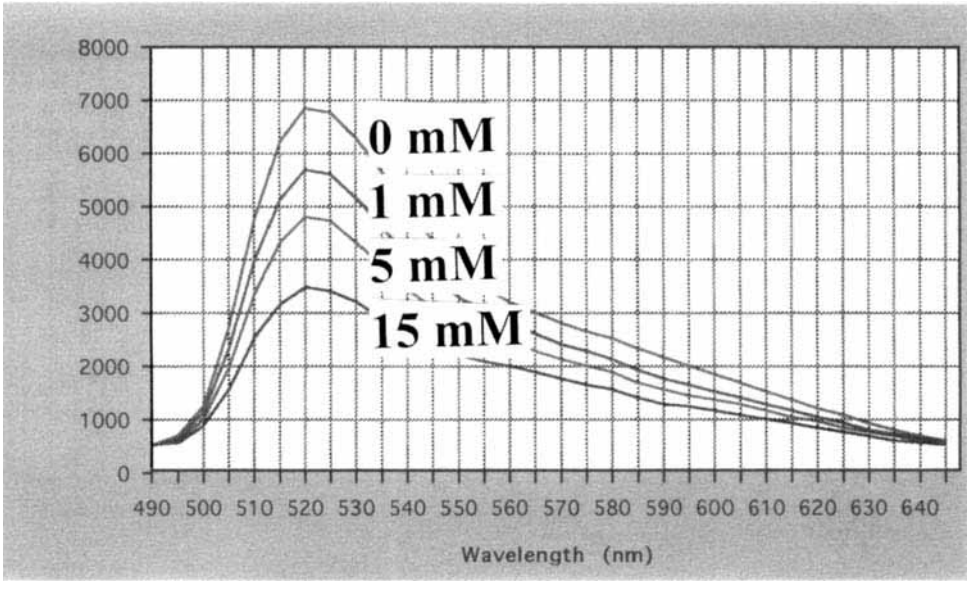

FIG.7. Spectra of the double-labeled substrate HHα1-S (500 nM), as shown in FIG.6 at 25°C and the indicated Mg concentrations (0, 1, 5 and 15 mM).

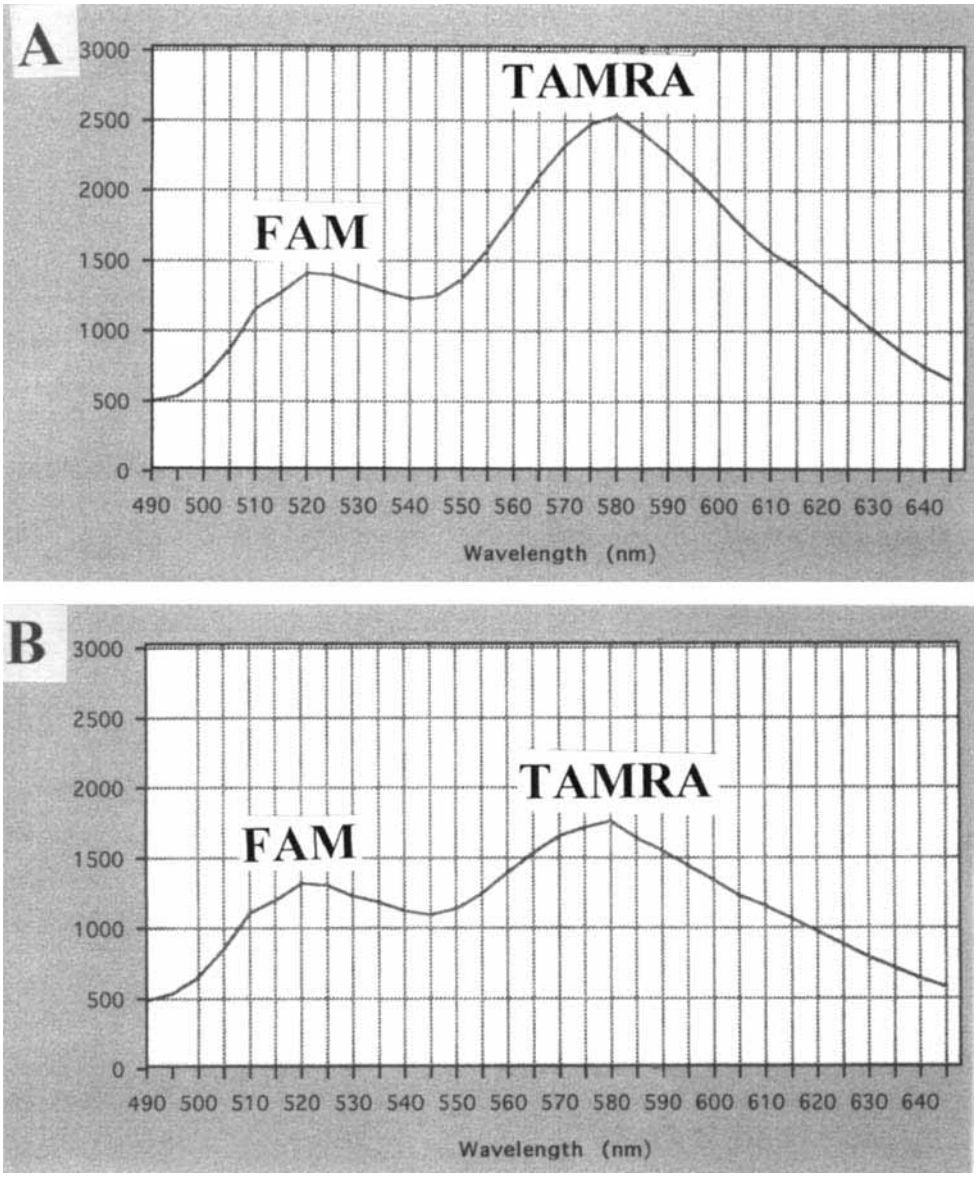

FIG.8. Incubation of the double-labeled substrate RNA (500 nM) with the structure shown in FIG.9. Panel A: At the beginning; Panel B: at the end of a 40 min incubation at 25°C.

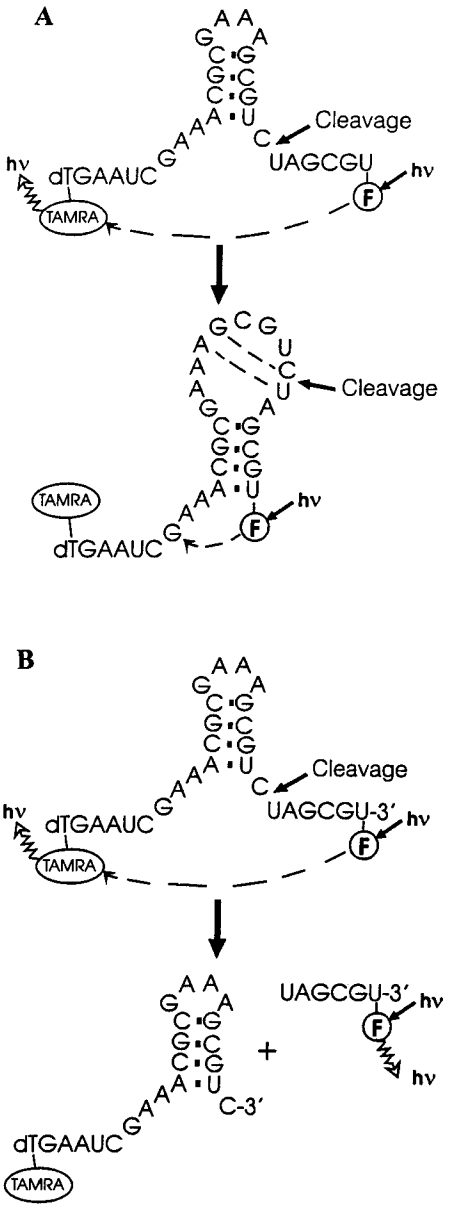

FIG.9. PanelA: Rearrangement of the substrate RNA as possible explanation for the observation in FIG.8. Panel B: Anticipated ribozyme cleavage reaction that should be monitored by FRET disruption.

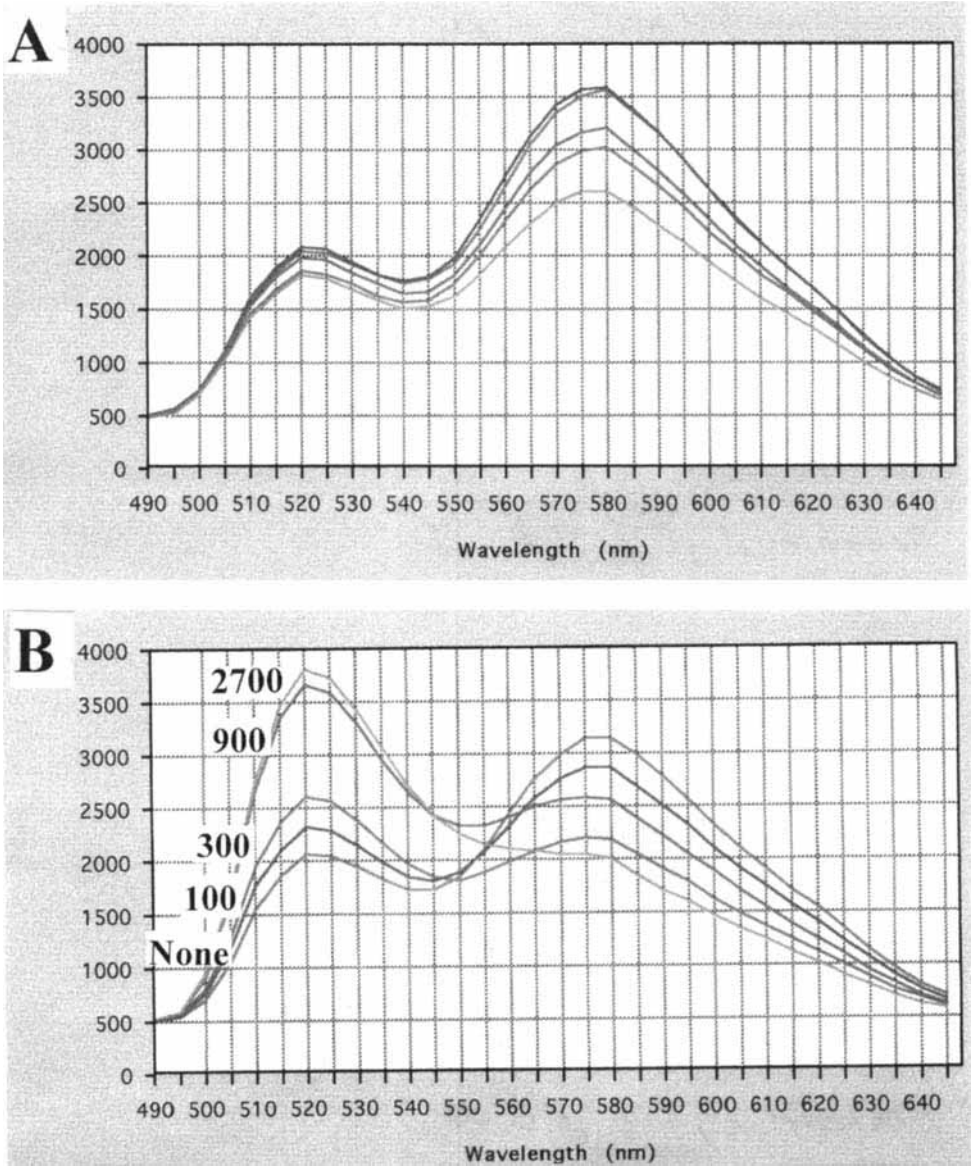

FIG.10. Incubation with 20 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5) and 10% ethanol at 37°C for 40 min. 500 nM substrate (FIG.9B) and a range of 54 nM to 2 nM ribozyme; total amounts of ribozyme per reaction tube are indicated (fmoles). Panel A: Begin, no significant differences between samples; Panel B: End of the incubation.

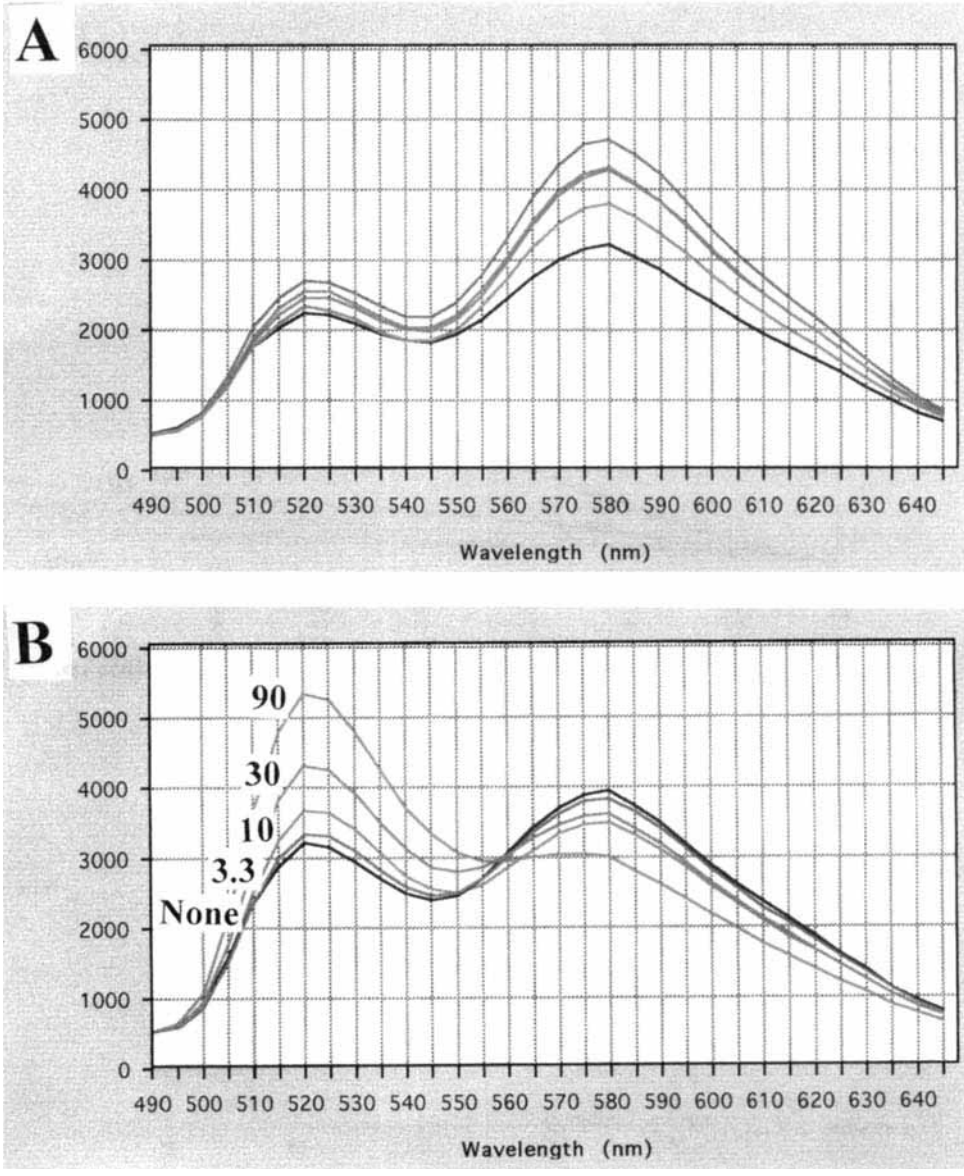

FIG.11. Incubation with 400 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.5) and 10% ethanol at 37°C for 200 min. 500 nM substrate (FIG.9B) and a range of 1.8 nM to 0.066 nM ribozyme; total amounts of ribozyme per reaction tube are indicated (fmoles). Panel A: Begin, no significant differences between samples; Panel B: End of the incubation.

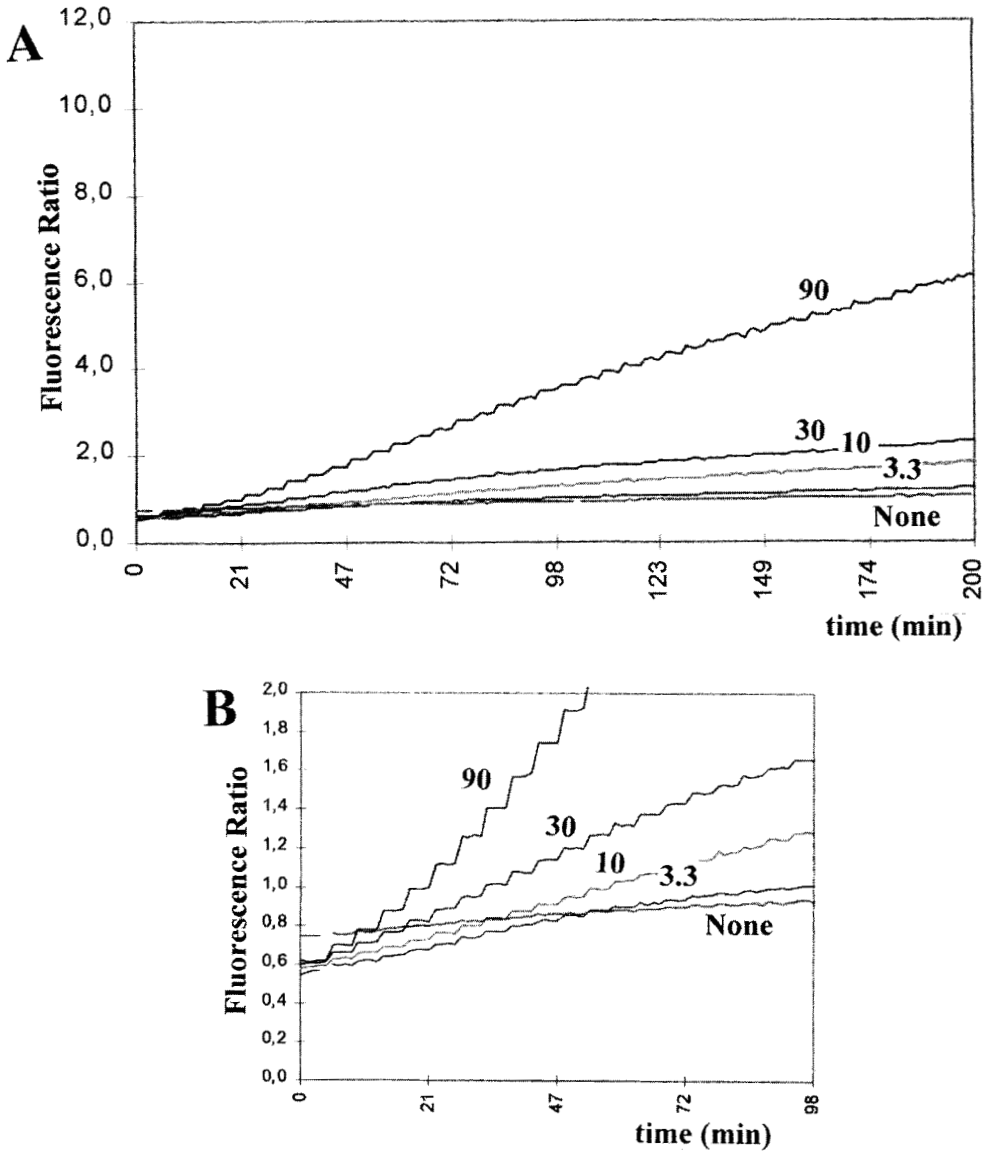

FIG.12. Alternative display of the data shown in FIG.11. Change of fluorescence ratios (signal for FAM divided by signal for TAMRA) for various ribozyme amounts versus time. Panel A: overview; Panel B: enlarged section.

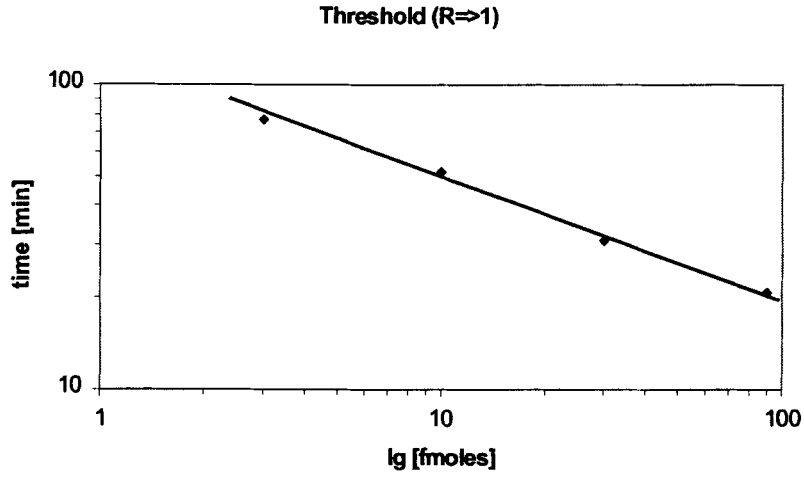

FIG.13. Time elapsed (min) until the fluorescence ratio reaches the value of 1.0 (FIG.12) is plotted versus the corresponding ribozyme amount (fmoles). Display in double logarithmic scale.

(iii) Possibly, fluorescent dyes enhance RNA interactions (6 base pair stem in FIG.1, 2; alternative stem in FIG.9, 10).

(iv) For the first time we have shown for multiple turnover conditions: Liberation of small fluorescent RNA products can be conveniently monitored in real time.

(v) FRET can be used as a sensitive tool for the quantitative detection of ribozyme sequences.

(vi) The high flexibility in the construction of ribozyme sequences and the extremely high specificity [6] allow specific sequence detection without need for stringent hybridization conditions.

EXPERIMENTAL

The large group I ribozyme was obtained by in vitro transcription from a plasmid template [4]. All other RNAs were transcribed from synthetic DNA templates [6] or chemically synthesized and obtained from NAPS GmbH, Göttingen. Transcription reactions and RNA purifications were performed as described [7]. Ribozyme reactions were performed in 50 µl and conditions were used as previously described for the group I [4] or the hammerhead ribozyme [5]. Any changes in the reaction conditions are indicated, and RNA concentrations are given for individual experiments.

Real time FRET measurements were performed with the Sequence Detection System ABI PRISM 7700. In addition, denaturing polyacrylamide gels were analyzed with a STORM imager (Molecular Dynamics) using the phosphorimager option for ^{32}P radioactivity, the blue mode for fluorescein (excitation at 450 nm, emission at 520 nm) or the red mode for Cy-5 fluorescence (excitation at 635 nm, emission at 670 nm).

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