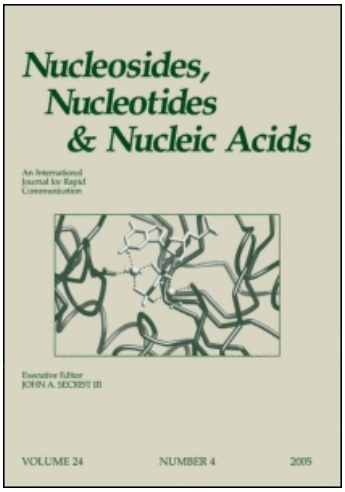

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Using Fluorescence Resonance Energy Transfer (FRET) for Measuring 2-5A Analogues Ability to Activate RNase L

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USING FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) FOR MEASURING 2-5A ANALOGUES ABILITY TO ACTIVATE RNase L

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Abstract. The development of a method for measuring the ability of 2-5A analogues to activate the cleavage of an oligoribonucleotide substrate by RNase L is described. This method is based on fluorescence resonance energy transfer. The method is easily performed with 96-well plates, allowing for quantitative high-throughput analyses of 2-5A analogues under different reaction conditions.

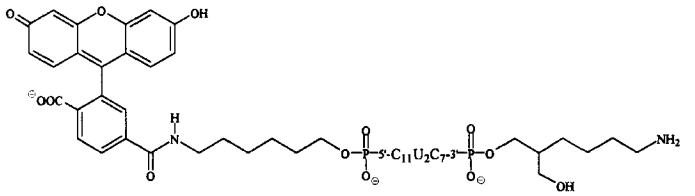
Introduction. The enzyme RNase L is a uniquely controlled endoribonuclease that exists in cells of higher vertebrates, and plays an important role in the cellular defense against viruses. Activation of RNase L by short 2',5'-linked oligomers of adenosine (2-5A) leads to RNA degradation and thus to an inhibition of protein synthesis in virally infected cells.

We have exploited the properties of RNase L for use in antisense therapeutics by conjugating a 5'-monophosphorylated 2',5'-oligoadenylate (2-5A) to an antisense oligonucleotide [1,2]. This strategy relies on the specific binding of the 2-5A antisense chimera to a chosen RNA and subsequent activation of the human RNase L, which then cleaves the targeted mRNA.

To better understand the activity of this enzyme and to optimize 2-5A-antisense oligonucletide development, a quantitative assay for RNase L activity is needed. Carroll et al. [3,4] used a cell-free system in which recombinant human RNase L was used to cleave oligoribonucleotides with mainly a single cleavage site, in particular $C_{11}U_2C_7$. In those studies, the substrate was radiolabeled and the reaction samples were analyzed by gel electrophoresis. While this method yielded useful kinetic data, it was limited by the manual rate of sampling.

Our method is based on a phenomenon called fluorescence resonance energy transfer (FRET). FRET involves the quenching of a fluorescent species by energy transfer to a nearby acceptor. A fluorescent substrate was generated by attaching a fluorescein dye as a photon donor to the 5'-end and a tetramethylrhodamine as an acceptor to the 3'-end of an oligoribonucleotide substrate. When this substrate is cleaved by RNase L, its fluorescence intensity is altered, which can be detected conveniently by using a 96-well plate microplate fluorometer.

Synthesis. For the synthesis of fluorescein-5'p-$C_{11}U_2C_7$-3'p-rhodamine (**2**) at first a 5'-fluorescein labeled oligonucletide **1** was assembled on a DNA synthesizer starting with



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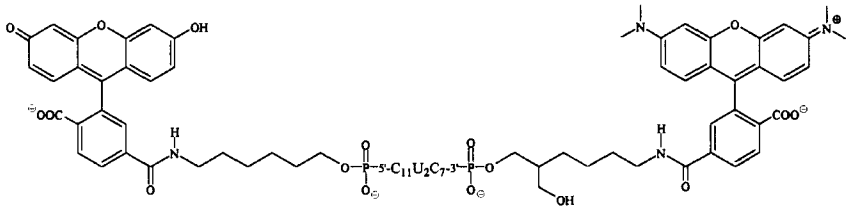

(2)

3'-DMT-C_6-Amine-On CPG as solid support. Standard RNA synthesis was followed by the attachment of the 5'-fluorescein using the commercially available 5'-fluorescein phosphoramidite. This resulted in a C-6 3'-amino modified fluorescein-5'p-$C_{11}U_2C_7$-3'p (**1**). **1** was treated for 2 h with tetramethylrhodamine *N*-hydroxysuccinimide ester. Excess dye was removed through size exclusion chromatography and the rhodamine labeled oligonucletide **2** was freed from unreacted **1** by anion exchange HPLC.

Fluorescence Measurements. Fluorescent measurements were performed using a 96-well plate fluorometer. Samples were analyzed using an excitation wavelength of 485 nm and detecting the emission at 538 nm. Addition of the subsrate **2** to the RNase L-containing buffer with $(p5'A2')_3$ as activator caused the fluorescence of the reaction mixture to rise, eventually becoming constant at a value of about 2.4-3.0 times the starting value.

Results and Discussion. It is known from Correl's investigations that the oligoadenylate-induced RNase L cleavage of the substrate $rC_{11}U_2C_7$ leads mainly to two products, C_7 and $C_{11}UUp$. By monitoring the cleavage of **2** and $rC_{11}U_2C_7$ with HPLC, we were able to demonstrate that **2** is cleaved at the same position as $rC_{11}U_2C_7$, and that the cleavage rates are similar for both compounds indicating that **2** is a good replacement substrate. Since the reaction progress as measured by fluorescence change, correlated very well to the values obtained with HPLC, we can conclude that the FRET assay yields kinetic data that are comparable to data obtained from the radiolabeling protocol reported by Carrol.

Conclusion. The FRET methodology is a useful way to screen 2-5A analogues for their abilities to activate RNase L in vitro. The results are as quantitative as radiolabeling approaches but less labor-intensive, allowing high-throughput screening of large numbers of 2-5A analogues.

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