

## 형광 염료로 인한 리보짐의 비활성화

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### Inactivation of the Ribozyme with Fluorescent Dyes

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#### INTRODUCTION

The Kin.46 kinase ribozyme was selected for the transfer of the thiophosphate from ATP- $\gamma$ -S to its own 5' hydroxyl end in the presence of oligonucleotide effector which is complementary to its 3' primer binding sequence (PBS) used in the amplification steps during the original selection for activity.<sup>1,2</sup> Omitting the oligonucleotides reduces the observed catalytic rate constant ( $k_{\text{obs}}$ ) by  $10^3$  to  $10^6$ -fold, indicating that the deoxyoligonucleotide effector is necessary for its full catalytic activity. The activator helix formed by the PBS and the oligo effector is connected by a 5nt "linker" region to the substrate-binding internal guide sequence and stabilizes a long-range base-pairing interaction between the 5 nucleotides of the linker and those closer to the catalytic core. According to our results, the activator helix is thought to stabilize the active conformation of the ribozyme by stabilizing the interaction between the linker and complementary nucleotides within the active site.<sup>3,4</sup>

Fluorescence resonance energy transfer (FRET) which is distance-dependent interaction between electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a

photon, has been used to get the information for the conformational change of RNA.<sup>5-8</sup> It is prerequisite to monitor the catalytic activity of ribozymes after the dye labeling for the fluorescence measurement because it can affect its catalytic activity. In this research, RNA species was prepared by labeling two different terminal sites of a kinase RNA with two fluorescent dyes such as a donor (Cy3) and an acceptor (Cy5).

#### RESULTS AND DISCUSSION

As the large single-strand region which has 20 nucleotides, joining the seven nucleotides to the rest of the ribozyme can be severed or omitted to yield trans-acting enzyme, ribozyme103 version derived from the Kin. 46 by the internal truncations has four different strands: a 7 nucleotide RNA substrate (7-mer), a 24 nucleotides "upper" strand (up), a 52 nucleotides "lower" strand (lw) and an activating oligomer (AO) with 18 nucleotides (Fig. 1). For an up strand, Cy3 was incorporated to the 5'-end during transcription with the class II promoter using AMP-dye-AMP as a primer. Cy5-labeled AO was purchased. It was analyzed that the four-stranded ribozyme assembled with a Cy3-up strand and a Cy5-AO18 folded predominantly into

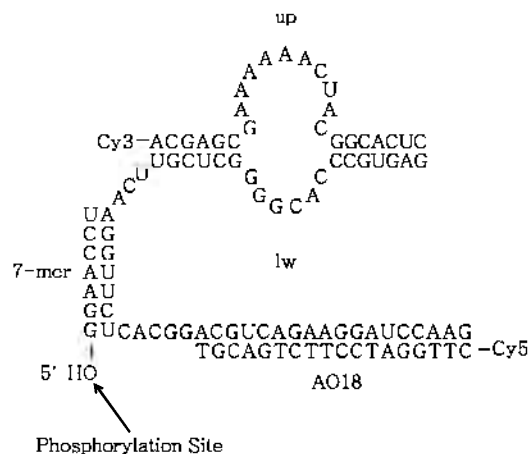


Fig. 1. An ribozyme103 derived from the Kin.46 self-thiophosphorylating ribozyme by internal deletions has 4 strands: a 7-mer, an upper (up) strand, a lower (lw) strand and an activating oligomer (AO) with 18 nucleotide. For an up strand, Cy3 was incorporated to the 5'-end during transcription with the class II promoter using AMP-dye-AMP as a primer. Cy5-labeled AO was purchased.

a single, active conformation after renaturation by native gel electrophoresis.<sup>5</sup> For the comparison of the catalytic activities of ribozymes, APM ([N-acryloylamino)phenyl] mercuric chloride) – PAGE (polyacrylamide gel electrophoresis) was used as a useful means which analyze thiolated or thiophosphorylated RNA by the strong interaction between mercury and sulfur (Fig. 2).<sup>9-11</sup> The mobility of RNA that carry thiophosphate monoester is diminished, compared with non-thiophosphated one. This is the evidence of the strong interaction between mercury and sulfur. Disulfides don't interact with the mercury in the gel matrix with APM.<sup>10</sup> Therefore, a ribozyme103, a truncated version of Kin.46 was incubated with ATP $\gamma$ S and the products were separated by PAGE using gels that contained APM. The initial velocities for the thiophosphorylation of ribozymes with and without two fluorescent dyes were plotted as a function of assay time. The observed rate constants are shown in Table 1. Ribozyme103 with both a Cy3 labeled up strand and a Cy5 labeled AO18 was almost 20 times less active ( $k_{\text{obs}} = 0.0025 \text{ min}^{-1}$ ) than the corresponding ribozyme103 without fluorescent dyes ( $k_{\text{obs}} = 0.0462 \text{ min}^{-1}$ ). This decrease of ribozyme activity with

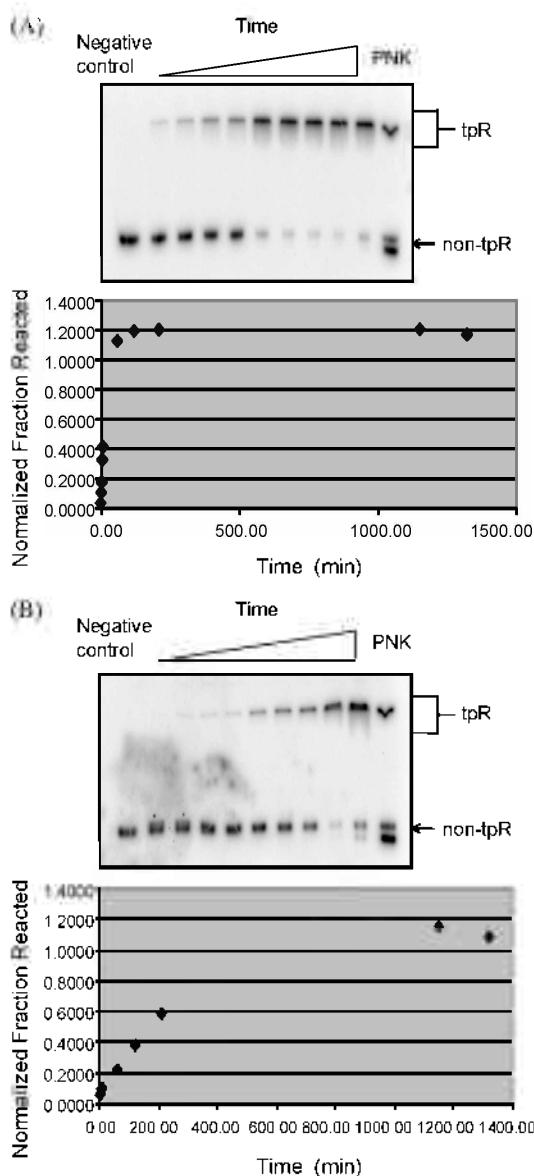


Fig. 2. Kinetic assay of dye-labeled ribozyme. The thiophosphorylation reaction of a ribozyme composed of a 7-mer, an upper (up) strand, a lower (lw) strand and an activating oligomer (AO) 18 (A) and a 7-mer, a Cy3-labeled upper (up) strand, a lower (lw) strand and a Cy5-labeled activating oligomer (AO) 18 (B) was initiated by addition of ATP $\gamma$ S to 10 mM at RT. Aliquots were removed at different times and the ribozymes thiophosphorylated (tpR) with ATP $\gamma$ S were separated from the nonthiophosphorylated ribozymes (non-tpR) within [(N-acryloylamino)phenyl] mercuric chloride (APM) polyacrylamide gel. PNK lanes treated with polynucleotide kinase were also applied to normalize each lane. The extent of thiophosphorylation was represented graphically.

Table 1. Comparison of ribozyme activities

	7-mer	7-mer
Ribozyme	up	upCy3
Composition	lw A018	lw A018Cy5
$k_{obs}$ ( $\text{min}^{-1}$ )	0.0462	0.0025

fluorescent dyes was also observed to ribozyme119 derivatives with both a Cy3-labeled lw strand and a Cy5-labeled AO18.<sup>12</sup> Although the hydrophobicity in fluorescent dyes is thought to affect the conformation of the ribozyme, the further study is needed for the reason of the decrease of the ribozyme activities by labeling the fluorescent dyes.

In conclusion, ribozyme103 derived from Kin.46 self-thiophosphorylating ribozyme by internal deletions and assembled from four different strands was terminally labeled with two fluorescent dyes of donor (Cy3) and acceptor (Cy5) and their activities for autothiophosphorylation were compared with APM-PAGE. The ribozyme with both a Cy3 labeled up strand and a Cy5 labeled AO18 was much less active than the corresponding ribozyme without fluorescent dyes.

## EXPERIMENTAL SECTION

**Kinetic assay of ribozyme.** An internally radio-labelled up strand using [ $\alpha$ -<sup>32</sup>P] UTP, a 7-mer and a lw strand of ribozyme, and an activating DNA oligomer were heated in KCl/Pipes buffer (200 mM KCl in 150 mM Pipes-KOH, pH 7.0) at 90 °C for 2 min and allowed to cool to RT (~21 °C). These were adjusted to a final concentration of 50 mM MgCl<sub>2</sub> and preincubated for 15 min at RT. The thiophosphorylation reaction was initiated by addition of ATPγS to 10 mM at RT. Aliquots were removed at different times (2 min, 5 min, 8 min, 10 min, 110 min, 230 min, 340 min, 1300 min and 1450 min) and the reaction was quenched with 94% formamide, 30 mM EDTA (pH 8.0) containing xylene cyanol and bromophenol blue. Thiophosphorylated ribozymes were separated from the nonthiophosphorylated by electrophoresis in 6% polyacrylamide gel with APM in 90 mM Tris-borate (pH 8.3) and

2.5 mM EDTA containing 7M urea. Dried gels were exposed to storage phosphor screens and imaging (Molecular Dynamics). The extent of thiophosphorylation was calculated by dividing the radioactivity in the product band (retained at the top of the APM layer) by the sum of reacted and unreacted bands. The data were fit to a kinetic equation. The first-order rate of thiophosphorylation ( $k_{obs}$ : observed rate constant) was calculated by fitting to  $f_t = (f_{\infty} - f_0) (1 - \exp(-k_{obs}t))$ , where  $f_t$  is the fraction normalized at time  $t$ .

**Preparation of dye-labeled strand.** An AMP-Cy3-AMP primer for transcription was kindly provided by Dr. Faqing Huang. An A residue was added to the 5' end of an up strand to allow the efficient transcription with the class II promoter (5'-TAAT-ACGACTCACTATT-3') by T7 RNA polymerase. In vitro transcription reaction with the class II promoter was performed at 30 °C for 2 ~ 4 hrs. Buffer composition was as follows; 40 mM Tris-Cl, pH 8.0, 5 mM DTT, 6 mM MgCl<sub>2</sub>, 2 mM spermidine, 0.01% TritonX-100, 0.25 mM ATP, 1 mM each of UTP, GTP and CTP, 2 mM dye, 0.05 ~ 0.5 μM dsDNA containing the T7 class II promoter, 500 units of T7 RNA polymerase per 100 μL reaction and 10 ~ 20 units of RNase inhibitor per 100 μL reaction.

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