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단신 (Notes)

Effect of Terminal Labeling of Fluorescent Dyes on Ribozyme Activity

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INTRODUCTION

The Kin. 46 kinase ribozyme was selected for the transfer of the thiophosphate from ATP-γ-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.[1,2] Addition of the deoxyoligonucleotide effector is necessary for its 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-paring interaction between the 5 nuclotides 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.[3,4]

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.[5-8] So FRET would be a good method to study the nature of the activator helix stabilization of this kinase ribozyme. The labeling step of two terminal sites of an RNA with fluorescent dyes such as donor (Cy3) and acceptor (Cy5), respectively, is a prerequisite for FRET. Since dye labeling can affect the ribozyme activity, this research was performed to examine how much dye labeling affects the catalytic activity of ribozymes.

RESULTS AND DISCUSSION

Ribozyme119 derived from the Kin. 46 by the internal truncations have 3 strands; left high (LH) strand, lower (lw) strand and activating oligomer (AO) with 18 nucleotides (*Fig.* 1). For lw strand, dyes (cy3 and cy5) were incorporated to the 5'-end during transcription with the class II promoter using AMP-dye-AMP as a primer. Since dye labeling of ribozyme can affect the activity, its activity should be compared with the original ribozyme without any fluorescent dye before measuring FRET. For the comparison of ribozyme activity, 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).[9-11] The mobility of RNA that carry thiophosphate monoester is diminished, when compared with non-thiophosphated one. This is the evidence of strong interaction between mercury and sulfur since disulfides don't interact with the mercury in the gel matrix with APM.[10] The observed rate constants for the thiophosphorylation of ribozymes with and without fluorescent dyes are shown in *Table* 1.

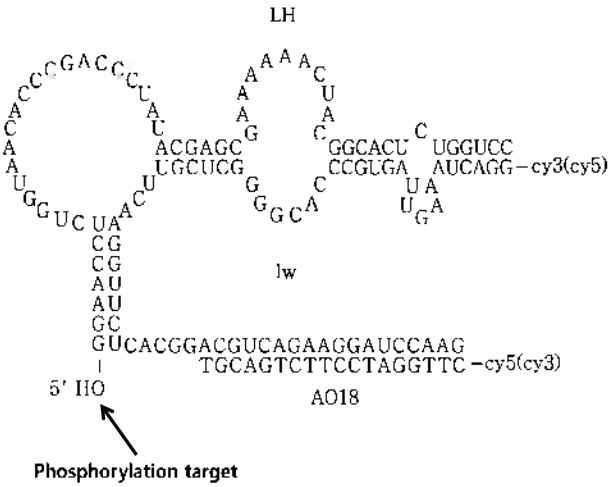

***Figure* 1.** Ribozyme119 derived from the Kin. 46 self-thiophosphorylating ribozyme by internal deletions have 3 strands; left high (LH) strand, lower (lw) strand and activating oligomer (AO) with 18 nucleotide. For lw strand, dyes (cy3 and cy5) were incorporated to the 5'-end during transcription with the class II promoter using AMP-dye-AMP as a primer.

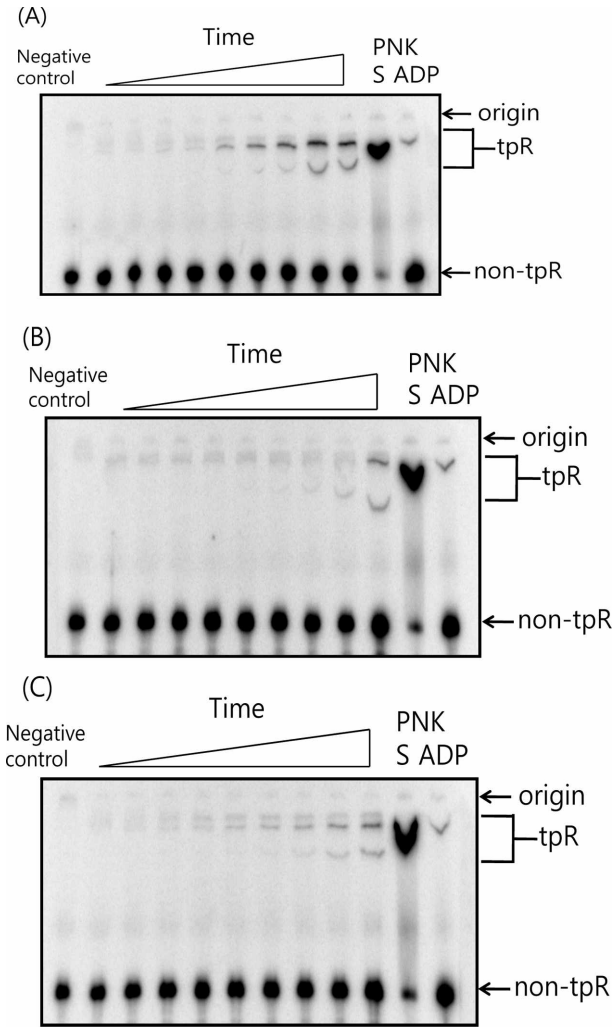

***Figure* 2.** Kinetic assay of dye-labeled ribozyme. The thiophosphorylation reaction of a ribozyme composed of left high (LH) strand, lower (lw) strand and activating oligomer (AO) 18 (A); left high (LH) strand, cy3-labeled lower (lw) strand and cy5-labeled activating oligomer (AO) 18 (B); and left high (LH) strand, cy5-labeled lower (lw) strand and cy3-labeled activating oligomer (AO) 18 (C), was initiated by addition of ATPgS to 10 mM at RT. Aliquots were removed at different times and ribozymes thiophosphorylated (tpR) with ATPγS were separated from nonthiophosphorylated ribozymes (non-tpR) within APM-polyacrylamide gel. PNK lanes treated with polynucleotide kinase were also applied to normalize each lane.

Without fluorescent dyes, ribozyme119 is active (k_{obs} = 0.0002 min^{-1}). Dye-labeled ribozyme with both cy5 labeled lw strand and cy3 labeled AO18 is also active (k_{obs} = 0.00024 min^{-1}) but the ribozyme with both cy3 labeled lw strand and cy5 labeled AO18 is almost inactive (k_{obs} = 5×10^{-5} min^{-1}), similar to the ribozyme103 assembled with four different strands.[12] At this point, we don't know the reason for the difference of ribozyme activities by only switching the positions of cy3 and cy5, for which further study is needed. Therefore the ribozyme with both cy5 labeled lw strand and cy3 labeled AO18 will be used for further FRET analysis.

***Table* 1.** Comparison of ribozyme activities

Ribozyme Composition	LH119 lw119 AO18	LH119 lw119cy3 AO18cy5	LH119 lw119cy5 AO18cy3
K_{obs} (min^{-1})	0.0002	5×10^{-5}	0.00024

In conclusion, a ribozyme119 derived from Kin. 46 self-thiophosphorylating ribozyme by internal deletions were terminally labeled with two fluorescent dyes of donor (Cy3) and acceptor (Cy5) and their activities were compared by APM-PAGE. The ribozyme with both cy5 labeled lw strand and cy3 labeled AO18 was as active as a ribozyme without any fluorescent dye but the ribozyme with both cy3 labeled lw strand and cy5 labeled AO18 was inactive.

EXPERIMENTAL SECTION

Kinetic Assay of Ribozyme

Internally radiolabelled LH strand using [α-^{32}P] UTP, lw strand of ribozyme, and 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 $MgCl_2$ 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 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 APM 6% polyacylamide gel 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. 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-Cy5-AMP primer for transcription was kindly

provided by Dr. Faqing Huang. An A residue was added to the 5' end of low strand to allow efficient transcription with class II promoter (5'-TAATACGACTCACTATT-3') by T7 RNA polymerase. *In vitro* transcription reaction with 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 $MgCl_2$, 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 uM dsDNA containing the T7 class II promoter, 500 units of T7 RNA polymerase per 100 uL reaction and 10-20 units of RNase inhibitor per 100 uL reaction. Dye-labeled AO was purchased.

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