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Notes

Dependence of Ribozyme Activity on the Positions of Fluorescent Dyes

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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] Omitting the oligonucleotides reduces the observed catalytic rate constant (k_{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 both PBS and the oligo effector, is connected to the substrate-binding internal guide sequence by a 5nt(nucleotide) "linker" region and this helix stabilizes a long-range base-paring interaction between the 5nt of the linker and sequence 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) 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. FRET has been used to get the information for the conformational change of RNA.[5–8] So FRET would be a good method to know the nature of the activator helix stabilization of this kinase ribozyme. In this research, ribozyme119 with four strands was derived from Kin.46 ribozyme by internal deletions because the labeling step of two terminal sites of RNA with fluorescent dyes such as donor (Cy3) and acceptor (Cy5), respectively, was prerequisite to FRET and it was monitored how dye labeling affects the catalytic activity of ribozymes.

RESULTS AND DISCUSSION

As the large single-strand region which has 20 nucleotides in Kin.46 ribozyme joins the seven nucleotides to the rest of the ribozyme and can be severed or omitted to yield trans-acting ribozyme, ribozyme119 versions derived from the Kin. 46 by the internal truncations of the large loop have four different strands; 7 nucleotide RNA substrate (7-mer), 31 nucleotides "upper" strand (up), 63 nucleotides "lower" strand (lw) 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 (5'-TAATACGACTCACTATT-3') using AMP-dye-AMP as a primer. Dye-labeled AO was purchased. It was analyzed that the four-stranded ribozyme assembled with cy3(cy5)-lw strand and cy5(cy3)-AO18 folded predominantly into a single, active conformation after renaturation by native gel electrophoresis.[3] Since dye labeling of ribozyme derivative could affect the activity, its activity was compared with the original ribozyme without any fluorescent dye before measuring FRET. For the comparison of the catalytic activities

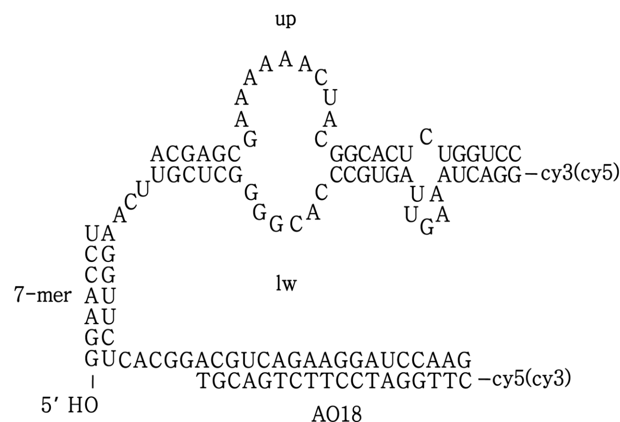

***Figure* 1.** Ribozyme119 derived from the Kin. 46 self-thiophosphorylating ribozyme by internal deletions has 4 strands; 7-mer, upper (up) strand, lower (lw) strand and activating oligomer (AO) with 18 nucleotide. For lw strand, dyes (cy3 and cy5) were incorporated to the 5'-end during transcription with the class II promoter using AMP-dye-AMP as a primer. Dye-labeled AO was purchased.

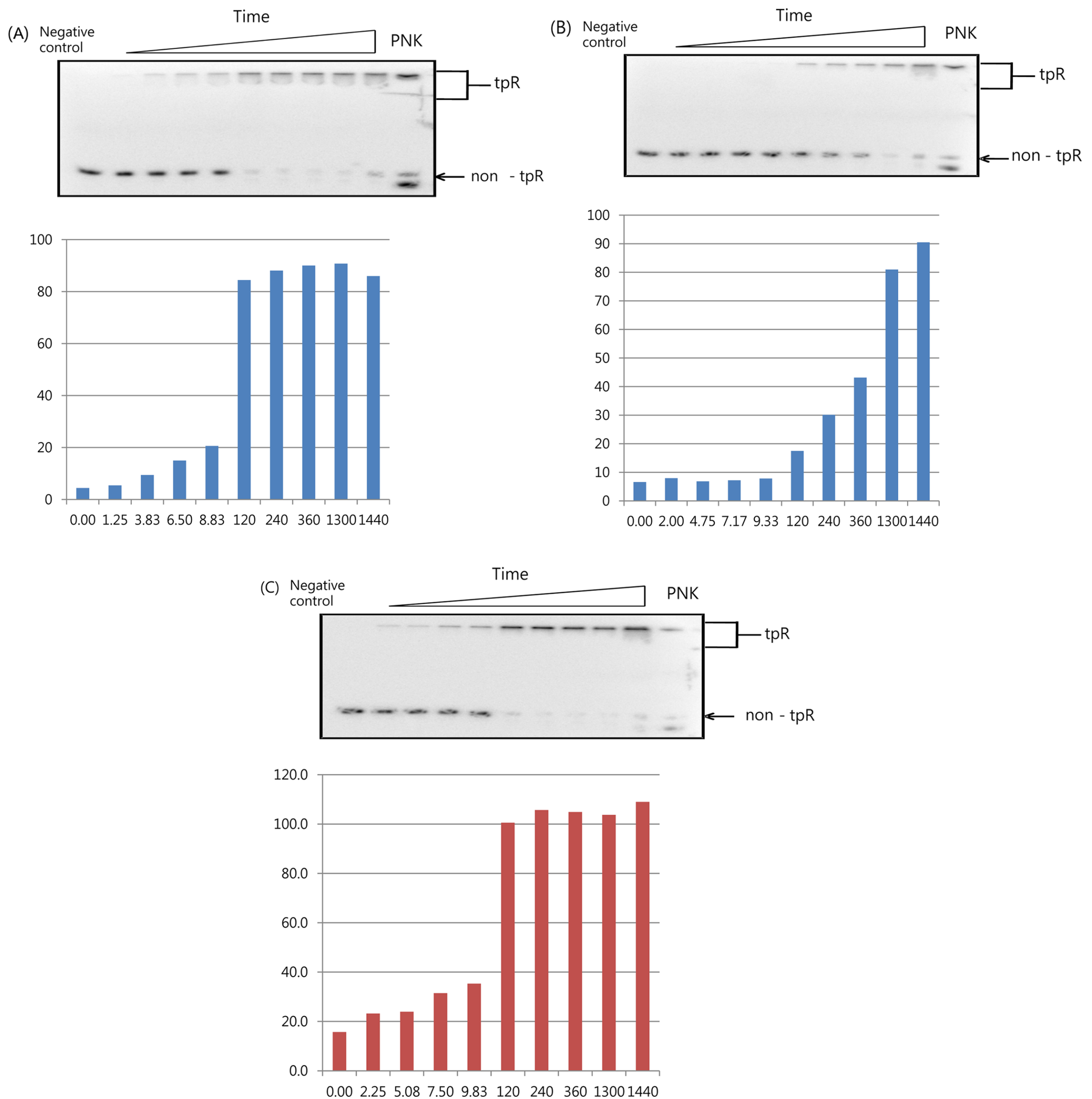

***Figure* 2.** Kinetic assay of dye-labeled ribozyme. The thiophosphorylation reaction of ribozyme composed of 7-mer, upper (up) strand, lower (lw) strand and activating oligomer (AO) 18 (A), 7-mer, upper (up) strand, cy3-labeled lower (lw) strand and cy5-labeled activating oligomer (AO) 18 (B), and 7-mer, upper (up) strand, cy5-labeled lower (lw) strand and cy3-labeled activating oligomer (AO) 18 (C) was initiated by addition of ATPγS to 10 mM at RT. Aliquots were removed at different times and the ribozymes thiophosphorylated (tpR) with ATPγS 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.

of ribozymes, APM ([(*N*-acryloylamino)phenyl] mercuric chloride) – PAGE (polyacrylamide gel electrophoresis) was used as a useful means which analyzed 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 the strong interaction between mercury and sulfur. Disulfides

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

Ribozyme Composition	7-mer up119 lw119 AO18	7-mer up119drd lw119 AO18	7-mer up119 lw119cy3 AO18cy5	7-mer up119 lw119cy5 AO18cy3
k_{obs} (min^{-1})	0.0185	0.0001	0.001	0.0189

don't interact with the mercury in the gel matrix with APM.[10] Therefore, ribozyme119, a truncated version of Kin.46 was incubated with ATPγS and the products were separated by PAGE using gels that contained APM. The observed rate constants for the thiophosphorylation of ribozymes with and without fluorescent dyes are shown in *Table* 1. Without fluorescent dyes, ribozyme119 derivative is active (k_{obs} = 0.0185 min^{-1}). Dye-labeled ribozyme with both cy5 labeled lw strand and cy3 labeled AO18 is also active (k_{obs} = 0.0189 min^{-1}) but the ribozyme derivative with both cy3 labeled lw strand and cy5 labeled AO18 is almost 20 times less active (k_{obs} = 0.001 min^{-1}) than the original ribozyme without any dye or the ribozyme derivative with cy5 labeled lw strand and cy3 labeled AO18. At this point, we don't know the reason for the difference of ribozyme activities by only switching the positions of cy3 and cy5, which the further study is needed for. Therefore the ribozyme with both cy5 labeled lw strand and cy3 labeled AO18 will be used for further FRET analysis.

In conclusion, ribozyme119 derived from Kin.46 self-thiophosphorylating ribozyme by internal deletions and assembled from four different strands were 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 cy5 labeled lw strand and cy3 labeled AO18 was as active as ribozyme without any fluorescent dye but the ribozyme with both cy3 labeled lw strand and cy5 labeled AO18 was almost inactive.

EXPERIMENTAL SECTION

Kinetic Assay of Ribozyme

An internally radiolabelled up strand using [α-^{32}P] UTP, 7-mer and 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 was quenched with 94% formamide, 30 mM EDTA (pH 8.0) containing xylene cyanol and bromophenol blue. Thiophosphorylated ribozymes were separated from the non-thiophosphorylated 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 the storage phosphor screens and imaging. The extent of thiophosphorylation was estimated 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 A residue was added to the 5' end of low strand using an AMP-Cy5-AMP primer 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.

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