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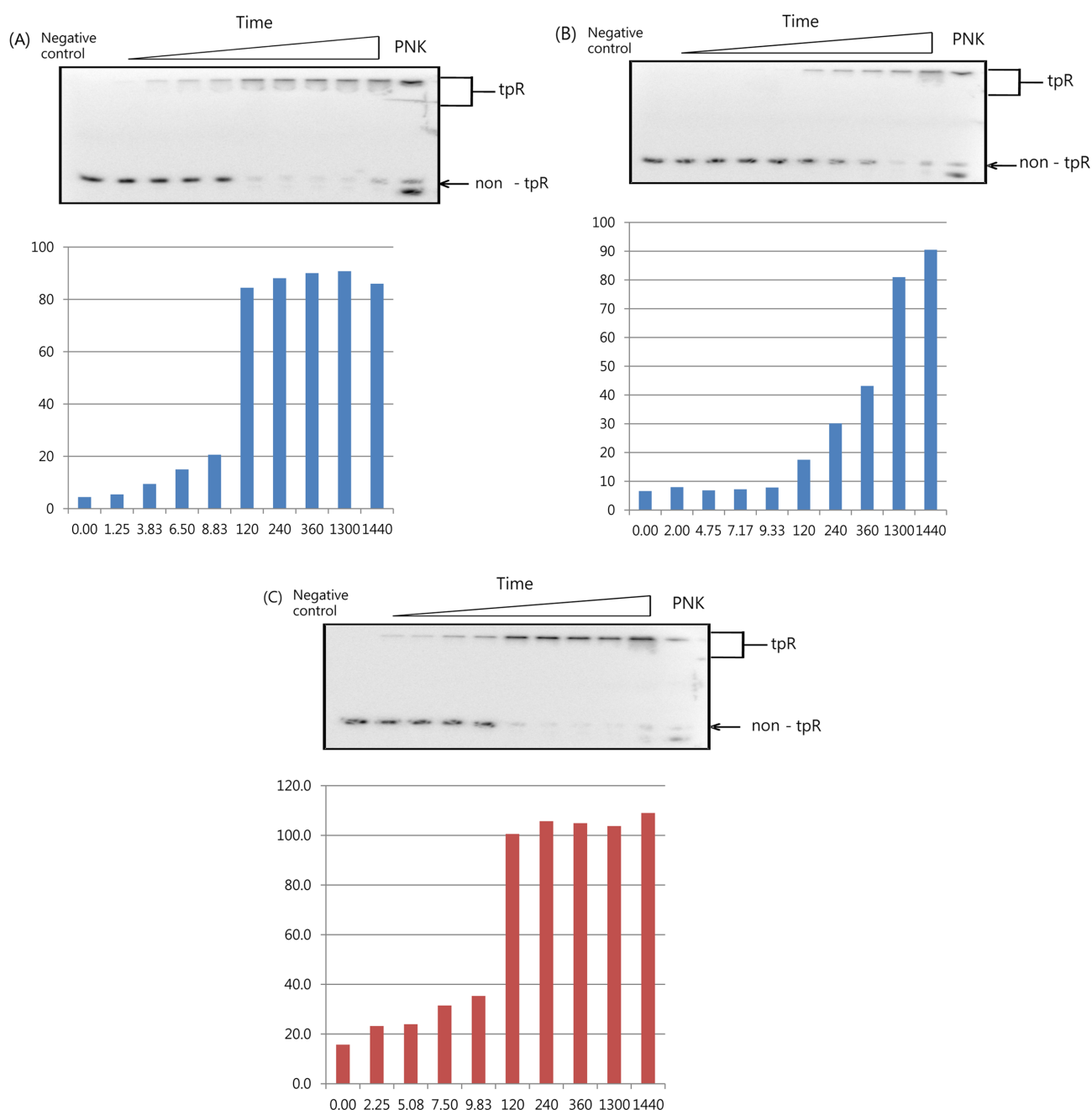


Figure 2. Kinetic assay of dye-labeled ribozyme. The thiophosphorylation reaction of ribozyme composed of 7-mer, upper (up) strand, lower (lw) strand and activating oligomer (AO) 18 (A), 7-mer, upper (up) strand, cy3-labeled lower (lw) strand and cy5-labeled activating oligomer (AO) 18 (B), and 7-mer,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

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

	7-mer	7-mer	7-mer	7-mer
Ribozyme	up119	up119drd	up119	up119
Composition	lw119	lw119	lw119cy3	lw119cy5
	AO18	AO18	AO18cy5	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 ATP γ S 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 ($k_{\text{obs}} = 0.0185 \text{ min}^{-1}$). Dye-labeled ribozyme with both cy5 labeled lw strand and cy3 labeled AO18 is also active ($k_{\text{obs}} = 0.0189 \text{ min}^{-1}$) but the ribozyme derivative with both cy3 labeled lw strand and cy5 labeled AO18 is almost 20 times less active ($k_{\text{obs}} = 0.001 \text{ min}^{-1}$) 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 -³²P] 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 MgCl₂ and preincubated for 15 min at RT. The thiophosphorylation reaction was initiated by addition of ATP γ S 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% polyacrylamide 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 $f_t = (f_{\infty} - f_0)(1 - \exp(-k_{\text{obs}}t))$, 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 MgCl₂, 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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