Restoration of Ribozyme Activity by Switching the Positions of Cy3 and Cy5 in the Ribozymes Inactivated with the Fluorescent Dyes

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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 and the 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 5 nucleotides "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]

The fluorescence measurement including Fluorescence resonance energy transfer (FRET) has been used to get the information for the conformational change of RNA.[5-8] It is prerequisite to monitor the catalytic activity of ribozyme after the dye labeling for the fluorescence measurement because it can affect its catalytic activity. In this work, two RNA species were prepared by labeling two different terminal sites of kinase ribozyme with two fluorescent dyes such as a donor (Cy3) and a acceptor (Cy5).

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 a trans-acting enzyme, two ribozyme119 versions were derived from the Kin.46 by the internal truncations. One version has three different strands; a 57 nucleotides "left high" strand (LH), a 62 nucleotides "lower" strand (lw) and an activating oligomer (AO) with 18 nucleotides and the other has four different strands; a 7 nucleotide RNA substrate (7-mer), a 30 nucleotides "upper" strand (up), a "lower" strand (lw) and an activating oligomer (AO) with 18 nucleotides (Fig. 1). For an lw strand, two dyes, Cy3 and Cy5 were incorporated to the 5'-end during the transcription reaction with the class II promoter for T7 RNA polymerase using AMP-dye-AMP as a primer.[9,10] Dye (Cy3 or Cy5)-labeled AO was purchased. For the comparison of the 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.[11-13] The mobility of RNA that carries thiophosphate monoester was diminished, compared with non-thiophosphated one. This is the evidence of strong interaction between mercury and sulfur. Disulfides don't interact with the mercury in the gel matrix with APM.[12] Therefore, ribozyme119 derivatives were incubated with ATPγS and the products were separated by PAGE using gels that contained APM (Fig. 2). The observed rate constants for the thiophosphorylation of ribozymes with

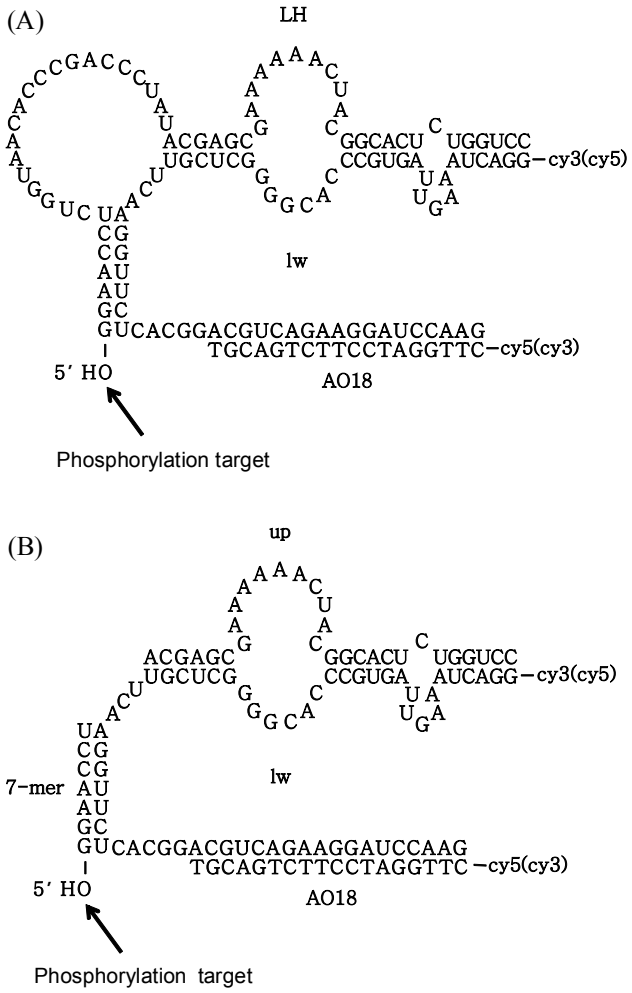

Figure 1. Ribozyme119 derivatives from the Kin.46 self-thiophosphorylating ribozyme by internal deletions has 3 strands; a left high (LH) strand, a lower (lw) strand and an activating oligomer (AO) with 18 nucleotide (A), and has 4 strands; a 7-mer, an upper (up) strand, a lower (lw) strand and an activating oligomer (AO) with 18 nucleotide (B). For a 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.

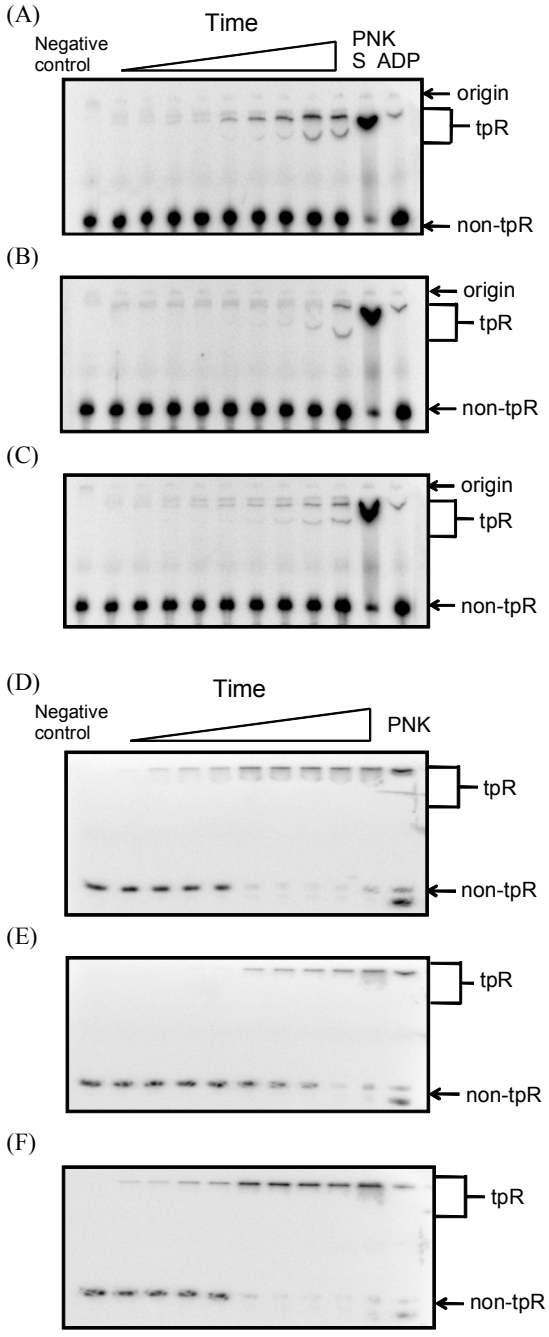

Figure 2. Kinetic assay of dye-labeled ribozyme. The thiophosphorylation reaction of a ribozyme composed of a left high (LH) strand, a lower (lw) strand and an activating oligomer (AO) 18 (A), a left high (LH) strand, a Cy3-labeled lower (lw) strand and a Cy5-labeled activating oligomer (AO) 18 (B) and a left high (LH) strand, a Cy5-labeled lower (lw) strand and a Cy3-labeled activating oligomer (AO) 18 (C) [or composed of a 7-mer, an upper (up) strand, a lower (lw) strand and an activating oligomer (AO) 18 (D), a 7-mer, an upper (up) strand, a Cy3-labeled lower (lw) strand and a Cy5-labeled activating oligomer (AO) 18 (E), and a 7-mer, an upper (up) strand, a Cy5-labeled lower (lw) strand and a Cy3-labeled activating oligomer (AO) 18 (F)] was initiated by the 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.

Table 1. Comparison of ribozyme activities

three-strand ribozymes			
Ribozyme Compositin	LH119 lw119 AO18	LH119 lw119cy3 AO18cy5	LH119 lw119cy5 AO18cy3
k_{obs} (min^{-1})	0.0002	5×10^{-5}	0.00025
four-strand ribozymes			
Ribozyme Compositin	7-mer up119 lw119 AO18	7-mer up119 lw119cy3 AO18cy5	7-mer up119 lw119cy5 AO18cy3
k_{obs} (min^{-1})	0.0185	0.001	0.0189

and without two fluorescent dyes are shown in Table 1. A three-strand ribozyme with both a Cy3-labeled lw strand and a Cy5-labeled AO18 was 4 times less active ($k_{obs} = 5 \times 10^{-5}$ min^{-1}) than the corresponding, three- strand ribozyme without fluorescent dyes ($k_{obs} = 0.0002$ min^{-1}) but a three-strand ribozyme with both a Cy5-labeled lw strand and a Cy3-labeled AO18 was as active ($k_{obs} = 0.00024$ min^{-1}) as the corresponding, three-strand ribozyme without dyes. It is interesting that one ribozyme species with two dyes which was less active than the corresponding, non-dye ribozyme restored its activity to that of the corresponding, non-dye ribozyme by only switching the positions of two dyes, Cy3 and Cy5. This restoration of the ribozyme activity was also observed in the four-strand ribozyme. A four-strand ribozyme derivative with both a Cy3-labeled lw strand and a Cy5-labeled AO18 was almost 20 times less active ($k_{obs} = 0.001$ min^{-1}) than the corresponding, four-strand ribozyme derivative without the fluorescent dyes ($k_{obs} = 0.0185$ min^{-1}) but a four-strand ribozyme with both a Cy5-labeled lw strand and a Cy3-labeled AO18 was as active ($k_{obs} = 0.0189$ min^{-1}) as the corresponding, four-strand ribozyme without the dyes. The further study is needed for the reason of the restoration of the ribozyme activities by only switching the positions of Cy3 and Cy5. The ribozyme whose activity was restored with both the Cy5-labeled lw strand and the Cy3-labeled AO18, is recommended for FRET analysis to know the nature of the activator helix stabilization of this kinase ribozyme.

In conclusion, ribozyme119 derivatives from Kin.46 self-thiophosphorylating ribozyme by internal deletions were prepared with two fluorescent dyes of a donor (Cy3) and a acceptor (Cy5), and their activities were compared with APM-PAGE. One ribozyme species with both the Cy3-labeled lw strand and the Cy5-labeled AO18 which is less active than the corresponding, non-dye ribozyme, restored its activity to that of the corresponding, non-dye ribozyme by only switching the positions of two dyes, Cy3 and Cy5.

Experimental Section

Kinetic assay of ribozyme. An internally radiolabelled LH strand using [α-^{32}P] UTP, a lw strand of ribozyme, and an activating DNA oligomer for a three-strand ribozyme (or an internally radiolabelled up strand using [α-^{32}P] UTP, a 7-mer and a lw strand of ribozyme, and an activating DNA oligomer for a four-strand ribozyme) 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 the 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% polyacylamide gel with APM in 90 mM Tris-borate (pH 8.3) and 2.5 mM EDTA containing 7M urea. The extent of thiophosphorylation was estimated by exposure to storage phosphor screens and imaging (Molecular Dynamics). 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 a low strand to allow efficient transcription with class II promoter (5'-TAATACG ACTCACTATT-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 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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