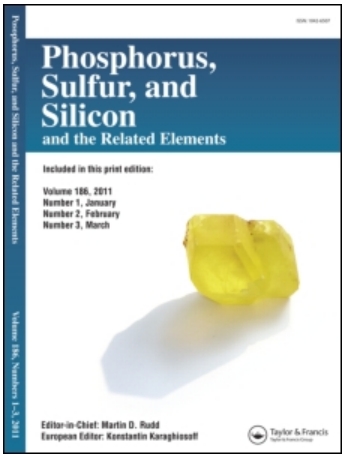

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Intracellularly Active Ribozymes in the Post-Genome Era

Hiroaki Kawasaki[a]; Kazunari Taira[b]
[a] Department of Chemistry and Biotechnology, School of Engineering, University of Tokyo, Hongo, Tokyo [b] Gene Discovery Research Center, National Institute of Advanced Industrial Science and Technology, Tsukuba Science City, Japan

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INTRACELLULARLY ACTIVE RIBOZYMES IN THE POST-GENOME ERA

Hiroaki Kawasaki[a] *and Kazunari Taira*[b]
Department of Chemistry and Biotechnology, School of Engineering, University of Tokyo, Hongo, Tokyo[a] *and Gene Discovery Research Center, National Institute of Advanced Industrial Science and Technology, Tsukuba Science City, Japan*[b]

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Artificial protein-RNA hybrid ribozymes with an unwinding activity were created. Since the novel hybrid ribozymes can attack any site within mRNA, libraries can be made of the hybrid ribozymes with randomized binding arms and thus be introduced into cells. This represents a new paradigm of powerful ribozyme technology that can enjoy many unique and exciting uses for various purposes in the post-genome project era, including applications for discovery of novel functional genes associated with specific important phenotypes and targeted elimination of expression of disease-causing genes in vivo *in gene therapy approaches.*

Keywords: Genes; hammerhead ribozymes; hybrid-ribozymes; maxizymes

INTRODUCTION

Hammerhead ribozymes are small and versatile nucleic acids that can cleave RNAs at specific sites. They consist of a catalytic domain surrounded by the substrate-binding arms called stems I and III.[1,2] The catalytic domain captures the catalytically indispensable Mg^{2+} ions. The ribozymes discussed below were developed as a result of studies to shorten the ribozyme.[3–9] In using the ribozyme in medical applications, ease of design and economics dictate that smaller size is preferable. Therefore, the design and construction of short ribozymes, namely the minizymes, have been attempted by many

Address correspondence to K. Taira, Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Tokyo 113-8656, Japan. E-mail: taira@chembio.t.u-tokyo.ac.jp

investigators;[10–13] unfortunately, such minizymes were found to have dramatically lower activities. However, we found that the minizyme completely lacking the stem-loop II region had activity essentially equivalent to the wild type.[3] Subsequent reaction kinetic analysis and NMR analysis indicated that the shortened ribozyme was essentially inactive as a monomer but exhibited extremely high cleavage activity as a dimer. We initially called this ribozyme a "dimeric minizyme" but latter renamed this structure "maxizyme." In addition, we also designed a heterodimeric system made of two different monomers, maxizyme left (MzL) and maxizyme right (MzR).[5–9] In this system, if MzL and MzR form a dimeric structure, the substrate will be cleaved.

The maxizymes were developed as a result of studies to shorten the hammerhead ribozyme. Because of the limitation for cleavable sequences on the target mRNA, conventional ribozymes would sometimes fail to possess precise cleavage specificity. To overcome this problem, an allosteric version (a maxizyme that functions as a dimer) was developed that displayed activity and specificity *in vivo*. The maxizymes, which function as dimers, have two substrate-binding regions. Such maxizymes form highly active dimers and successfully cleave various unwanted mRNAs both *in vitro* and *in vivo*.[4–9] We have taken advantage of this feature—that maxizymes can bind to two different target sites—to develop a system that can be used to inactivate gene expression. More than five custom-designed maxizymes have clearly demonstrated sensor functions, indicating that our technology might be broadly applicable in molecular biology and possibly in a clinical setting.

Despite extensive efforts, the efficiencies of ribozymes *in vivo* usually are not high enough to achieve the desired biological effect(s).[14] Successful gene inactivation by ribozymes *in vivo* depends strongly on the design of the expression vector. The design can determine both the level of expression and the half-life of the expressed ribozyme. In previous studies, we found that pol III-mediated expression of ribozymes as tRNA fusions resulted in highly expressed stable ribozymes.[15–17]

However, even these improved ribozymes were sometimes ineffective, probably because the ribozyme was unable to locate its target. One potential explanation for this ineffectiveness is that the rate-limiting step *in vivo* for the cleavage of phosphodiester bonds is the annealing/association of the ribozyme with its target site.[17] Furthermore, in a long RNA chain, significant numbers of target sites are not accessible to the ribozyme since they are hidden within secondary or tertiary structures. This problem is often critical in attempts to exploit ribozyme activity, in particular, *in vivo*.

To overcome the problem of accessibility, computer generated secondary structure predictions are typically used to identify targets most likely to have an open conformation. However, these predictions are often inaccurate because of unpredictable RNA-protein interactions that change the structure of RNA in cells. To circumvent this limitation, sometimes an unwieldly systematic approach involving huge numbers of candidate antisense molecules is employed.[14] To avoid being dependent on either of these approaches, we sought to develop a ribozyme that would be able to access any chosen target site independent of local secondary structure.[18]

RESULTS AND DISCUSSION

We reasoned that it would be useful to design a ribozyme that could recruit a protein that could in turn relieve any interfering secondary structure, thereby making any site accessible to the ribozyme. To accomplish this, we tried to link a ribozyme to an RNA helicase, proteins demonstrated to have nonspecific RNA binding, sliding, and unwinding activities. We introduced an RNA motif, the RNA helicase-binding motif (RBM), that interacts with RNA helicases *in vitro* and *in vivo*.[18] To investigate whether the protein that binds to the $tRNA^{Val}$-driven RBM-attached ribozymes has unwinding and cleavage activities, we performed *in vitro* cleavage assay by these ribozyme-protein complexes.

At first, we generated duplexes as substrates by hybridizing partial mRNAs and mixed with RBM-connected or -unconnected ribozyme-protein complexes. As shown in Figure 1, RBM-unconnected ribozymes did not unwind the duplexes and, thus they were unable to cleave the substrate. By contrast, RBM-attached ribozyme-protein complexes were clearly capable of unwinding and cleaving the substrate. However, the inactive ribozyme-protein complex (I-Rz2-RBM with a single G-to-A mutation at the catalytically important conserved nucleotide) could unwind duplexes but did not cleave the substrate. Thus, these results clearly demonstrate that RBM-attached ribozyme-protein complexes (hybrid-ribozymes) had two activities, i.e., unwinding and cleavage, and those unwinding activities were due to the RNA helicase. Thus, importantly, the novel hybrid-ribozymes could cleave inaccessible target sites that were not cleavable by conventional ribozymes.

Hybrid-ribozymes are able to cleave the target mRNA at any chosen site, regardless of the putative secondary or tertiary structure in the vicinity of the target site, and thus can be used for rapid identification of functional genes in the post-genome era.

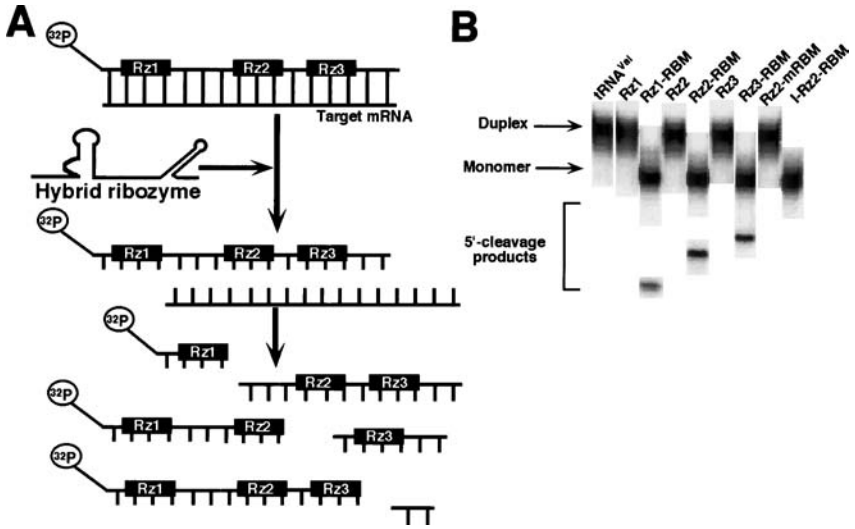

FIGURE 1 RBM-connected ribozyme-protein complexes had two activities, such as unwinding and cleavage *in vitro*. (A) Schematic representation of unwinding and cleavage assays *in vitro*. (B) Cleavage activity *in vitro* of RBM-connected or -unconnected ribozyme-protein complexes.

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