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Detection of mRNA in Mammalian Cells with a Split Ribozyme Reporter

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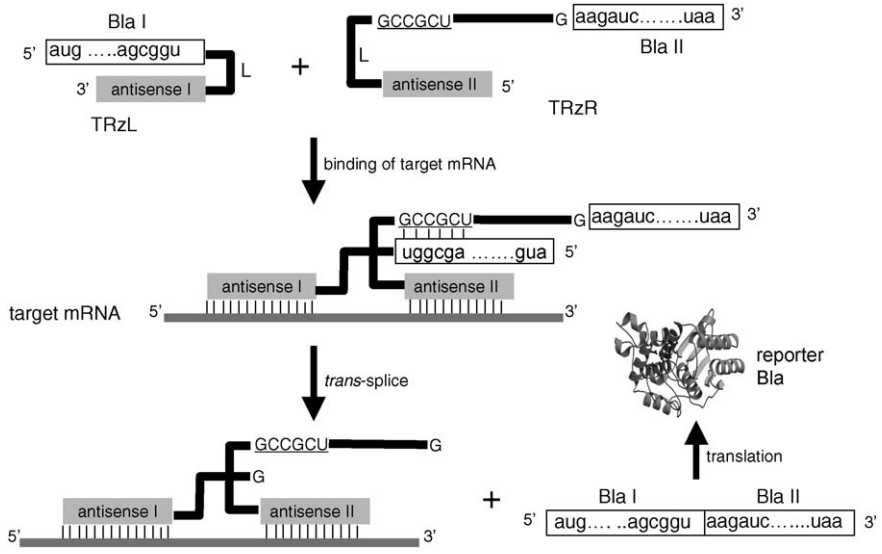
The detection of mRNA expression in vivo can reveal essential information about basic biology and disease processes. Current methods primarily involve the use of labeled (with fluorophores or radioactive isotopes) antisense oligomers, based on a one-to-one receptor–ligand type of interaction without robust signal amplification, such as molecular beacons.[1–7] Given that the copy number of a particular mRNA target per cell is generally ~50–1000,[8] it is challenging to detect such a small number of mRNA in vivo with current methods. Here we introduce a new strategy with a mechanism of signal amplification for sensing target mRNAs in mammalian cells. This strategy utilizes the *Tetrahymena* group I intron ribozyme as an RNA sensor.

Group I introns of the ciliated protozoan *Tetrahymena thermophila* have been shown to catalyze *trans*-splicing of mRNA molecules in mammalian cells in which the ribozyme splices an attached 3′ exon to a designated splice site on any chosen target RNA.[9–11] This property has been variously exploited for ribozyme-mediated repairing of mutant mRNA transcripts.[12–14] We were interested in exploiting this splicing activity to detect mRNAs in mammalian cells because it can potentially achieve robust signal amplification through enzymatic catalysis. Split-reporter technology based on protein (intein) splicing has been demonstrated with *Renilla* luciferase and green fluorescent protein in detecting protein–protein interactions.[15–18] The splicing activity of the *Tetrahymena* ribozyme may be similarly applied to develop a biosensor for mRNA detection.

We previously reported a *cis*-splicing ribozyme construct, Rz156, in which the *Tetrahymena* group I intron ribozyme was inserted into the coding sequence of the cDNA of the nonsecreted TEM-1 β-lactamase (Bla).[19] Based on this *cis*-splicing ribozyme, we devised a split-reporter strategy for the detection of target mRNA in cells (Scheme 1). In this design, Rz156 was split at the L1 loop into a pair of plasmid constructs—TRzL and TRzR—each carrying a part of the coding sequence of the Bla reporter and a fragment of the *Tetrahymena* ribozyme. Both TRzL and TRzR also carried an antisense sequence complementary to a target mRNA, and a six-nucleotide-long linker



Scheme 1. Schematic presentation of the strategy for detecting target mRNA with a split ribozyme reporter. Bla I and Bla II are the 5′ and 3′ fragments of Bla mRNA, respectively.

(L in Scheme 1) complementary to its counterpart on the other construct to assist the association of the split reporter constructs. TRzL and TRzR would not assemble and splice in the absence of the target mRNA; the interaction between the internal guiding sequence on TRzR (GCCGCU in Scheme 1) and the six nucleotides on TRzL (agcggu) is too weak to induce efficient splicing in vivo. The presence of the target mRNA will assemble TRzL and TRzR through sense–antisense base-pairing, generating a complete Bla reporter mRNA after splicing. The ligated Bla mRNA can be translated into the Bla enzyme, which can be readily detected with fluorogenic substrates. Therefore, in this strategy, one single binding event of target mRNA with the split ribozyme reporter can lead to the production of many copies of the reporter Bla enzyme by translation, and produce greatly amplified signals through enzymatic catalysis.

The mRNA of red fluorescent protein (DsRed) was first chosen to test our design. Figure 1 A outlines the targeting sites on the DsRed mRNA by the split ribozyme reporter. A series of TRzL and TRzR constructs was made with varied lengths of antisense sequence complementary to the DsRed mRNA. For example, TRzL60 and TRzR60 each have 60-nt antisense sequences targeting the DsRed mRNA from nt128 to 187

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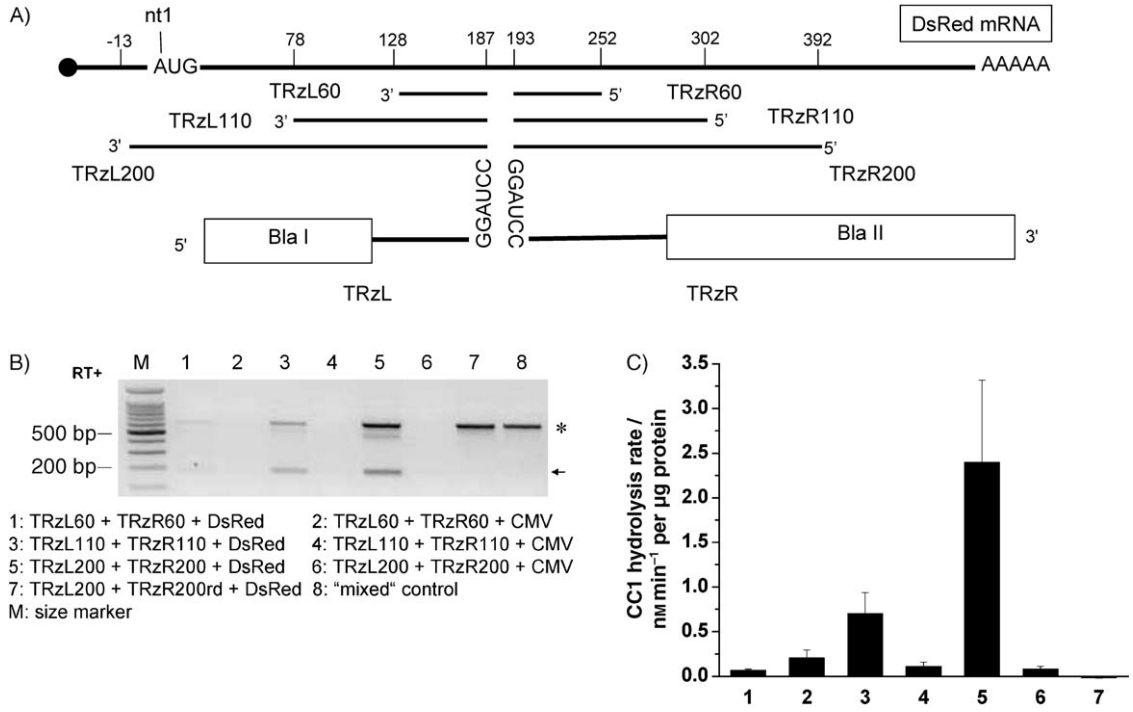

Figure 1. Detection of DsRed mRNA with the split reporter ribozyme. A) Targeting sites of three pairs of TRzL/TRzR: 60, 110, and 200 nucleotides. B) RT-PCR analysis of COS-7 cells transiently transfected with the indicated constructs. An arrow indicates the expected size of the splice product. C) CC1 assays of the reporter Bla activity of COS-7 cells transiently transfected with constructs 1–7 in (B).

and from nt193 to 252, respectively. The two target sequences were five nucleotides apart to avoid potential steric hindrance during the complex assembly.

COS-7 cells were transfected with these pairs of TRzL and TRzR constructs along with the DsRed vector expressing the target DsRed mRNA or an empty vector, and the extracted RNAs were analyzed by RT-PCR for splice products. A clearly detectable splice product of the appropriate size was seen only when construct pairs TRzL110/TRzR110 or TRzL200/TRzR200 were cotransfected in COS-7 cells along with DsRed (Figure 1B). Sequence analysis of the splice products confirmed that correct *trans*-splicing had occurred. It appears that there is an increase in the amount of spliced product produced by TRzL200 and TRzR200; this indicates that a stronger interaction between the target mRNA and the split reporter ribozyme enhances the efficiency.

A fair amount of larger-molecular-weight RT-PCR products was also observed (top bands marked by an asterisk in Figure 1B). Sequence analysis of this product revealed that it contained both the Bla fragments and ribozyme; this suggests that they might be strand-transferring products of reverse transcription. We have observed the same phenomenon before,[20] and strand transfer mediated by reverse transcriptase pausing could occur during reverse transcription.[21,22]

The observed *trans*-splicing was dependent on the splicing activity of the group I intron, because an inactive ribozyme mutant (TRzR200rd) with a point mutation G264A in the catalytic core of the group I intron could not induce *trans*-splicing of the tri-RNA complex (Figure 1B; lane 7). This single mutation scarcely perturbed the folding or stability of the ribozome but decreased catalytic efficiency.[23,24] A "mixed" control, in which total cellular RNAs from cells transfected separately with TRzL200 or TRzR200 or DsRed were mixed in vitro, produced no splice products (Figure 1B; lane 8); this suggests that target-dependent *trans*-splicing did not occur in a test tube during the RT-PCR reaction.

The activity of the translated Bla reporter from the splicing reaction was assayed by using a fluorogenic Bla substrate CC1.[25] The final Bla activity, determined as the rate at which it catalyzed the hydrolysis of CC1, was generally < 0.1 nM min^{-1} per µg of protein when TRzL/TRzR pairs were cotransfected with an empty vector, thus verifying that there was little Bla activity in the absence of the target mRNA (Figure 1C). In contrast, when TRzL110/TRzR110 or TRzL200/TRzR200 were cotransfected along with a DsRed vector, the rates were 0.7 and 2.4 nM min^{-1} per µg of protein, which correspond to 7- and 24-fold increases, respectively, compared with that of no target (Figure 1C). These results further confirmed that the split reporter ribozyme can be assembled and function under the guidance of a target mRNA in vivo.

We further tested the applicability of this split-reporter system to detecting a clinically and biologically important target—the p53 gene. p53 is one of the best known tumor-suppressor genes, and overexpression of dominant negative mutants of p53 has been observed in many human cancers.[26–28] The targeting scheme for the p53 gene with a single dominant negative mutation (p53DN) is shown in Figure 2A. This mutant was chosen for the study in order to avoid the apoptotic effects that would be caused by overexpression of wild-type p53. The construct pair p53TRzL200 and p53TRzR200

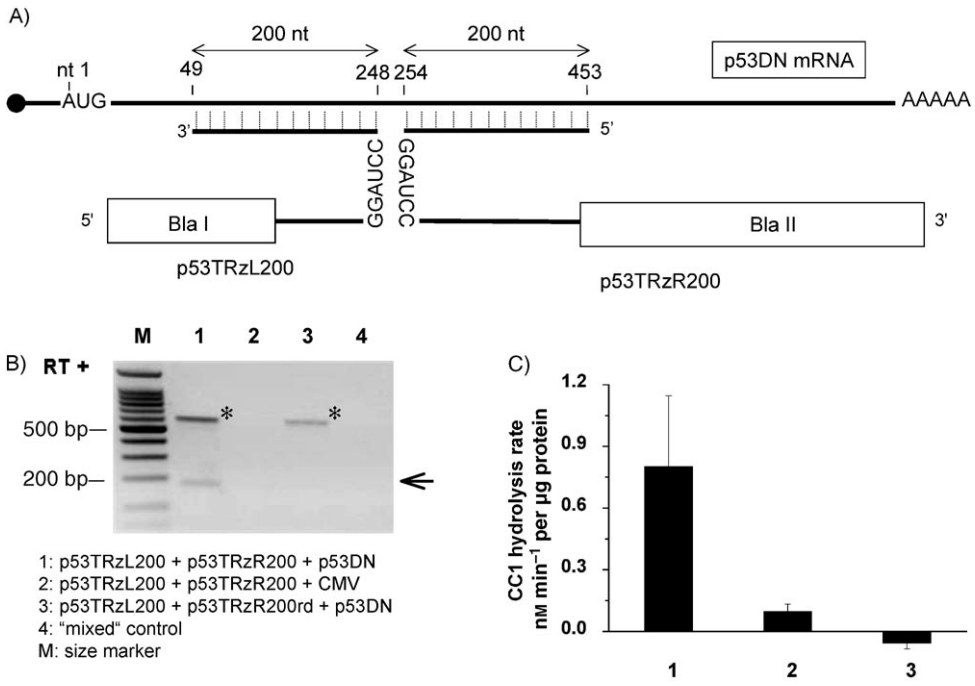

Figure 2. Detection of p53DN mRNA with the split ribozyme reporter. A) Targeting scheme of p53TRzL200/p53TRzR200. B) RT-PCR analysis of COS-7 cells transiently transfected with the indicated constructs. The expected size of the splice product is indicated by an arrow. Asterisks indicate the unspliced product derived from the tri-RNA complexes. C) CC1 assays of the Bla activity of COS-7 cells transiently transfected with constructs 1–3 in (B).

carried antisense sequences against the p53DN mRNA from nt 49 to 248 and from nt 254 to 453, respectively. p53TRzR200rd, an inactive ribozyme construct with a point mutation G264A, was constructed as a negative control. COS-7 cells were co-transfected with the plasmid vectors encoding p53TRzL200 and p53TRzR200, along with a plasmid encoding p53DN or an empty vector.

RT-PCR analysis revealed the spliced products at the expected size are found only when the *trans*-splicing vector pair was expressed along with the target p53DN gene (Figure 2B; lane 1). Sequencing of the spliced products confirmed that the correct splicing had occurred. As expected, a p53TRzL200/p53TRzR200rd pair did not produce the spliced band (Figure 2B; lane 3). No spliced band was observed in a "mixed" control either; this confirmed that *trans*-splicing did not take place during the RT-PCR reaction. The Bla activity of lysates extracted from cells transfected with p53TRzL200, p53TRzR200, and p53DN was 0.8 nM min^{-1} per µg of protein, which was about eight times higher than that in cell lysates with no target (Figure 2C). Since current targeting sites were not optimized, this result may be improved.

In conclusion, we present here the novel strategy of using a split ribozyme reporter to sense mRNA in mammalian cells, and have demonstrated its in vivo applicability with two mRNA targets. In principle, this strategy can be applied to any target RNA by selecting appropriate antisense sequences, and can be adapted to reporters other than Bla. The signal-amplification mechanism in this strategy might be important for successful detection and imaging of mRNAs in living cells and living subjects. Further development is ongoing to optimize this design towards this goal.

Experimental Section

Plasmid construction: All ribozyme constructs were derived from previously reported constructs pCMV-Sb211 or pCMV-TRz211.[29] To construct the plasmid pCMV-TRzL, Bam HI–Sal I fragment encoding an antisense sequence complementary to the target sequence of DsRed mRNA was generated by PCR and a subsequent enzyme digestion. These fragments were ligated to Bam HI/Sal I sites of the pCMV-Sb211. For the plasmid pCMV-TRzR, we prepared Sal I–Bam HI fragments containing an antisense sequence complementary to the target sequence by PCR. After enzyme digestion with Sal I and Bam HI and gel purification, they were ligated to Sal I/Bam HI sites of the pCMV-TRz211. The PCR primers used were the following:

LCA200S (5′-GGCGTCGACACCGGTCGCCACCATGGCCTCC-3′) and LCA200AS (5′-CGGGATCCGGGACAGGATGTCCCAGGCGAAGG-3′) for pCMV-TRzL200; LCB200S (5′-CGGGATCCTTCCAGTACGGCTCCAAGG-3′) and LCB200AS (5′-GGCGTCGACGAGGGGAAGTTCACGCCGATG-3′) for pCMV-TRzR200; LCL110S (5′-GGCGTCGACGTTCGAGATCGAGGGCGAGGGC-3′) and LCA200AS for pCMV-TRzL110; LCR110 AS (5′-GGCGTCGACCTTCTTGTAGTCGGGGATGTCGG-3′) and LCB200S for pCMV-RedTRzR110; LCL60S (5′-GGCGTCGACCCGTGAAGCTGAAGGTGACCAAG-3′) and LCA200AS for pCMV-TRzL60; LCR60 AS (5′-GGCGTCGACTCCTCGAAGTTCATCACGCGCTCC-3′) and LCB200S for pCMV-RedTRzR60. Similarly, for the construction of p53 targeting plasmids, we used LCLp53/200S primer (5′-GGCGTCGACGAAACATTTTCAGACCTATGG-3′) and LCLp53/200AS primer (5′-CGGGGATCCGCCGGTGTAGGAGCTGCTGG-3′) for pCMV-p53TRzL200. LCRp53/200S primer (5′-CGGGGATCCCTGCACCAGCCCCCTCCTGG-3′) and LCRp53/200AS primer (5′-GGCGTCGACGGGTGTGGAATCAACCCACAGC-3′) were used for pCMV-p53TRzR200. Ribozyme mutants with aborted splicing activity were generated from pCMV-TRz211D. pDsRed2-N1 and pCMV-p53mt135 (BD Biosciences Inc.) were used as target mRNAs. All plasmids were confirmed by DNA sequencing.

RT-PCR: COS-7 cells were seeded in a six-well dish at a density of 4.0×10^5 per well 18–24 h before transfection by Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, total RNAs were extracted by TRIzol (Invitrogen) according to the manufacture's instructions. Before RNA extraction, EDTA was added to TRIzol (at a final concentration of 45 mM) to inhibit ribozyme splicing activity. Total RNA (1 µg) was treated by DNaseI (Invitrogen). DNaseI-treated RNA (100 ng) was reverse transcribed by oligo dT primer (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen) in the presence of L-argininamide (10 mM). After RNase H treatment, the resultant cDNAs were amplified by the following protocol: 94 °C (5 min), 27 or 30 cycles of 94 °C (30 s), 58 °C (30 s),

72 °C (1 min), and final extension step at 72 °C for 7 min. Primers used were RT-1 forward primer (5′-CAGAAACGCTGGTGAAAG-3′) and RT-2 backward primer (5′-CGTCAATACGGGATAATACC-3′). PCR products were analyzed by electrophoresis in agarose gel (2%). The identities of the splice products were confirmed by sequence analysis.

CC1 assay: COS-7 cells were transfected with expression vectors (1.3 μg per well) by Lipofectamine 2000. Forty-eight hours after transfection, cells were collected by centrifugation at 2600 *g* for 1 min at room temperature. After the cells had been washed with PBS (800 μL; GIBCO/BRL), cell pellets were resuspended in phosphate buffer (0.1 M, 50 μL; pH 7.0). Cell lysates were prepared by three freezing and thawing cycles (freezing in dry ice/propan-2-ol for 10 min and thawing in a 37 °C water bath for 10 min). The supernatant was recovered by centrifugation at 16000 *g* for 2 min at 4 °C. Assays were conducted in 96-well black-wall microtiter plates (Corning). To measure the Bla activity, cell lysate (45 μL) and CC1 (1 mM, 5 μL) were mixed in each well. Fluorescence was measured by using 360 nm excitation and 465 nm emission at each time point in a SAFIRE microplate reader (TECAN, Research Triangle Park). Fluorescence data were converted to the hydrolysis rate based on a standard curve of coumarin, the fluorophore generated during the hydrolysis of CC1, and then normalized against total lysate protein contents determined by a Bradford assay (Bio-Rad).

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