

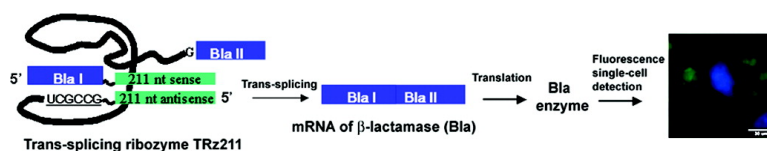
Communication

Single-Cell Detection of Trans-Splicing Ribozyme In Vivo Activity

Sumitaka Hasegawa, Jung W. Choi, and Jianghong Rao

J. Am. Chem. Soc., **2004**, 126 (23), 7158-7159 • DOI: 10.1021/ja049144u • Publication Date (Web): 18 May 2004

Downloaded from <http://pubs.acs.org> on February 23, 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

[View the Full Text HTML](#)

Single-Cell Detection of Trans-Splicing Ribozyme In Vivo Activity

Sumitaka Hasegawa, Jung W. Choi, and Jianghong Rao*

Department of Radiology & Bio-X Program, Molecular Imaging Program at Stanford, Stanford University,
300 Pasteur Drive, Stanford, California 94305-5344

Received February 16, 2004; E-mail: jrao@stanford.edu

The trans-splicing *Tetrahymena* ribozyme is derived from the self-splicing group I introns of *Tetrahymena thermophila*. It recognizes a target mRNA by its complementarity to its internal guide sequence (IGS) and cleaves the target mRNA into a 5' and a 3' fragment. The 5' fragment is subsequently ligated to an exon attached to its 3' end.^{1,2} Therefore, trans-splicing ribozyme is able to repair defective mRNA targets in a sequence-specific manner, and is a promising therapeutic agent for genetic diseases.³ There are examples that trans-splicing ribozymes can work in mammalian cells, but splicing efficiency and production of final proteins are not high enough for functional rescues.^{4–6} Improving ribozyme in vivo efficiency still remains a challenging task due to a relative paucity of information on what may enhance ribozyme reaction efficiency in the complex intracellular environment of mammalian cells. Alternatively, a systematic search from a library of ribozymes in the context of living mammalian cells would help discover improved trans-splicing ribozyme variants. In vitro selections for desired ribozyme variants and optimization of trans-splicing ribozymes in yeast have been documented previously,^{7,8} but a systematic mammalian-cell-based library screening is more desirable but not yet reported. Here we report an enzyme-based method that can detect the trans-splicing activity of *Tetrahymena* ribozyme in single living mammalian cells by fluorescence microscopy and flow cytometry, which should allow a single-cell-based systematic screening for trans-splicing ribozyme variants with improved in vivo efficiency.

Our method utilizes the 29 kDa TEM-1 isoform of β -lactamase (Bla), the product of the ampicillin-resistant gene *Amp^r*, as a reporter. We have previously applied it to detect the self-splicing activity of *Tetrahymena* group I intron ribozyme.⁹ To examine the feasibility of detecting the trans-splicing ribozyme activity in single living mammalian cells, we constructed a trans-splicing ribozyme with one part of Bla mRNA (Bla II) attached to its 3' end (Scheme 1). Its IGS was designed so that it recognized the other part of Bla

Scheme 1. Schematic Presentation of the Strategy for the Detection of Trans-Splicing Ribozyme Activity by Bla



mRNA (Bla I). Selection of Bla I and Bla II was based on our previously published work on cis-splicing ribozymes.⁹ The trans-splicing reaction then produced a complete mRNA of Bla, and the Bla activity can be readily detected by fluorogenic substrates CC1 and CCF2/AM.^{10,11}

It has been reported that extended flanking sequences at the 3' end of the substrate RNA and the 5' end of the trans-splicing ribozyme can enhance splicing activity.^{6,12} We initially added 38 nucleotides derived from the mRNA of a red fluorescent protein,

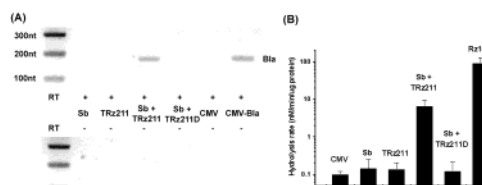


Figure 1. RT-PCR and CC1 assays of COS-7 cells transiently transfected with indicated constructs. (A) Reverse transcriptase (RT) was present in upper samples but not in lower. The expected splicing product Bla was as indicated. (B) Shown is the hydrolysis rate of CC1, determined from three independent transfections and normalized against total lysate protein contents in nM/min per μ g protein (in a log scale).

DsRed, to the 3' end of the substrate RNA and the corresponding antisense sequence to the 5' end of the trans-splicing ribozyme. Both constructs were cloned into a plasmid vector with a cytomegalovirus (CMV) promoter for expression in mammalian cells. After a transient cotransfection into COS-1 cells (a monkey kidney cell line) and incubation for 48 h, reverse transcription (RT)-PCR analysis of isolated total RNAs showed the desired spliced product. The Bla activity could be detected by CC1 assay in vitro but not in single cells even with 16 h of incubation of CCF2/AM. We therefore extended the flanking sequence to 211 nucleotides. The substrate target RNA was named Sb and the trans-splicing ribozyme as TRz211 (Scheme 1).

We first investigated whether TRz211 could trans-splice in vitro. Sb and TRz211 were prepared by in vitro transcription and mixed and incubated under the splicing conditions. The reaction mixture was analyzed by RT-PCR and the desired spliced product was detected with the expected size. Sequencing of the RT-PCR product confirmed that correct ligation took place.

We conducted RT-PCR analysis to examine whether trans-splicing between TRz211 and Sb occurred in mammalian cells. Both Sb and TRz211 were cloned into a plasmid vector with a CMV promoter for expression in mammalian cells. RT-PCR analysis of isolated total RNAs from a transient cotransfection of TRz211 and Sb into COS-7 cells (a monkey kidney cell line) showed a detectable RT-PCR product with the expected molecular weight. Sequence analysis of the product confirmed that accurate ligation occurred at the splice site and a correct Bla mRNA was produced (Figure 1A). As expected, single transfection of either construct did not produce any RT-PCR product. We constructed TRz211D, a catalytically inactive mutant of TRz211 with a mutation at the catalytic core of ribozyme (G264A). Consistent with previous in vitro studies,¹³ this single mutation resulted in no detectable RT-PCR products (Figure 1A). No amplified products were observed in the control where cells separately transfected with single vector were mixed shortly after lyses (data not shown). These data suggest that TRz211 ribozyme trans-spliced Sb and produced Bla in mammalian cells not during the RT reactions.

We assayed the Bla activity of cell lysates with CC1. The final Bla activity was measured by its rate in catalyzing the hydrolysis

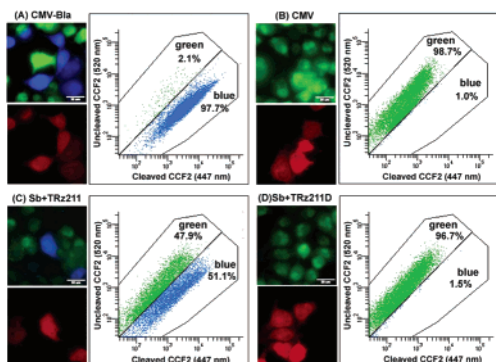


Figure 2. Fluorescence microscopy and FACS analyses of COS-7 cells transiently transfected with (A) CMV-Bla (positive control) (B) CMV (negative control), (C) Sb+TRz211, or (D) Sb+TRz211D. DsRed was used as a transfection marker in all samples. Each upper image is an overlay of frames captured at 530 nm (green emission) and 460 nm (blue emission); each lower image is DsRed emission at 605 nm excited at 546 nm. Cells with no Bla activity are green in upper images. The scale bar is 30 μ m. FACS analyses were performed on the DsRed-positive (transfected) cells. The percentages of Bla-positive (blue) and Bla-negative (green) cells (defined as blue/green ratios >1 and <1 , respectively) are indicated for each construct.

of CC1, which was 6.6 ± 3.1 nM/min/(μ g protein). This value was >60 -fold higher than that of CMV control construct. Neither single transfection of Sb or TRz211 nor cotransfection of Sb and TRz211D produced any significant Bla activity compared to that of the empty CMV vector (Figure 1B).

To detect the trans-splicing activity in single living cells, we stained transfected COS-7 cells with CCF2/AM and imaged them under a fluorescence microscope. CMV-Bla transfected cells emitted blue signals from cleaved substrates (Figure 2A). For empty-vector (CMV), transfected or nontransfected cells all emitted green light from uncleaved substrates (Figure 2B). Cotransfection of Sb, TRz211, and DsRed revealed that some DsRed-positive (transfected) cells showed blue emission after 3 h incubation of CCF2/AM, whereas DsRed-negative (nontransfected) cells emitted green due to lack of Bla activity (Figure 2C). For nontransfected cells, the blue/green ratio was low (<0.5), and for transfected cells some had a high blue/green ratio of >1.5 . The fact that all cells with high blue/green ratios also emitted red supports that their Bla activity came from the trans-splicing ribozyme. Controls of single transfection with either Sb or TRz211 did not produce any cells with a blue/green ratio >0.5 (data not shown). Cells cotransfected with the dead mutant TRz211D and Sb did not produce any blue signal either.

We quantified the trans-splicing activity in a large population of living cells by flow cytometry. COS-7 cells transfected with Sb, TRz211, and DsRed (as a cotransfection marker) were loaded with CCF2/AM and incubated for 3 h. We first used a fluorescence-activated cell sorter (FACS) to sort and collect about 10 000 DsRed-positive healthy cells for each construct. These cells were subsequently subjected to a second FACS analysis for the Bla activity. In the negative control (CMV empty vector), 99% cells fluoresced green, and in the positive control (CMV-Bla) over 97% of transfected cells displayed a blue/green ratio of >1 (Bla-positive) (Figure 2, A and B). When cotransfected with DsRed, Sb, and TRz211D, only 1.5% of cells emitted blue signals (Figure 2D). In contrast, 51.1% of cells transfected with Sb and TRz211 were Bla-positive (Figure 2C); clearly, its Bla activity was correlated to the trans-splicing activity of TRz211. The significant cell-to-cell heterogeneity emphasizes the importance of examining the trans-splicing reaction in single cells.

We have shown here for the first time that the trans-splicing activity of ribozymes in single living mammalian cells can be readily detected. More than 50% of transfected cells produced the final functional protein. The final enzyme activity was about 7% of that of our previously reported cis-splicing ribozyme Rz156 (Figure 1B). In considering the length of the extended flanking sequence, this activity is still remarkable because, in the complex of Sb and TRz211, the introduced additional sequence would be 422-nt long, greater in length than native *Tetrahymena* group I intron ribozyme (413 nt). As we have shown in our previous study of cis-splicing ribozymes, a large insertion at the L1 loop may decrease the ribozyme activity; for example, a mutant with an insertion of 84 nucleotides has about 12% Bla activity of Rz156.⁹

This enzyme-based single-cell detection of trans-splicing activity is compatible with the high-throughput flow cytometry and thus may be applied to screen large libraries of ribozymes in living cells to maximize in vivo production of proteins resulting from correct trans-splicing reaction. Although in this study we used an exogenous mRNA as the substrate, this strategy could be extended to detect trans-splicing between endogenous mRNA targets and ribozymes in single living cells. This system should then offer a unique advantage over other selection techniques such as antibiotic resistance for its ability of performing both negative (for minimal background in the absence of target RNAs) and positive selections (for maximal activity in the presence of target RNAs).

In summary, we present here a highly sensitive enzyme-based method for single-cell detection of trans-splicing ribozyme activity in vivo. This method can be potentially applied in performing in vivo optimization of trans-splicing ribozymes and in using trans-splicing ribozymes to target and image endogenous mRNAs. Results from these studies may be translated into a variety of biological and medical applications such as probing the structure–function relationship of ribozymes, repairing defective genes in inherited diseases, and detecting tumor-specific oncogenes.

Acknowledgment. We thank Lance Hultin at UCLA FACS facility for expert assistance with the FACS experiment and Dr. Roger Tsien at UCSD for generous gifts of COS-7 cells and CCF2/AM. This work was supported by Burroughs Wellcome Fund. S.H. was in part supported by a medical fellowship from the Sumitomo Life Insurance Welfare Services Foundation.

Supporting Information Available: Detailed experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Inoue, T.; Sullivan, F. X.; Cech, T. R. *J. Mol. Biol.* **1986**, *189*, 143.
- (2) Been, M. D.; Cech, T. R. *Cell* **1986**, *47*, 207.
- (3) Sullenger, B. A.; Gilboa, E. *Nature* **2002**, *418*, 252.
- (4) Rogers, C. S.; Vanoye, C. G.; Sullenger, B. A.; George, A. L., Jr. *J. Clin. Invest.* **2002**, *110*, 1783.
- (5) Lan, N.; Howrey, R. P.; Lee, S. W.; Smith, C. A.; Sullenger, B. A. *Science* **1998**, *280*, 1593.
- (6) Watanabe, T.; Sullenger, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8490.
- (7) (a) Beaudry, A. A.; Joyce, G. F. *Science* **1992**, *257*, 635. (b) Bartel, D. P.; Szostak, J. W. *Science* **1993**, *261*, 1411. (c) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818. (d) Guo, F.; Cech, T. R. *Nat. Struct. Biol.* **2002**, *9*, 855.
- (8) Ayre, B. G.; Kohler, U.; Turgeon, R.; Haseloff, J. *Nucleic Acids Res.* **2002**, *30*, e141.
- (9) Hasegawa, S.; Jackson, W. C.; Tsien, R. Y.; Rao, J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 14892.
- (10) Gao, W.; Xing, B.; Tsien, R. Y.; Rao, J. *J. Am. Chem. Soc.* **2003**, *125*, 11146.
- (11) Zlokarnik, G. N.; Paul, A.; Knapp, T. E.; Mere, L.; Burres, N.; Feng, L.; Whitney, M.; Roemer, K.; Tsien, R. Y. *Science* **1998**, *279*, 84.
- (12) Ayre, B. G.; Kohler, U.; Goodman, H. M.; Haseloff, J. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3507.
- (13) Michel, F.; Hanna, M.; Green, R.; Bartel, D. P.; Szostak, J. W. *Nature* **1989**, *342*, 391.

JA049144U