Selection of intracellularly active ribozymes in mammalian cells

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ABSTRACT
Ribozymes are expected to be useful as antiviral agents and powerful tools of functional analysis of unknown gene products in vivo. For use of ribozymes in vivo, they must be fully functional in the intracellular environment. Not all ribozymes selected in vitro would be expected to work in vivo, whereas ribozymes selected in the intracellular environment should retain their function in vivo. With the eventual aim of using ribozymes as antiviral agents or biological tools in mammalian cells, we then devised a novel selection system in mammalian cells of active ribozymes by targeting at a gene for the cyclin dependent kinase inhibitor (CDKI), p16INK4a. In this system, we found that p16INK4a-knockdown cells became malignant and they formed foci. In the mammalian system, we confirmed that the selected cells harbored the active ribozyme, indicating that our positive selection systems in vivo were operational.

INTRODUCTION
Ribozyme technologies appear to have potential as methods for suppressing the expression of a specific gene (1-3). Therefore, they could be powerful tools in gene therapy for some diseases caused by the aberrant gene expression, including diseases caused by infectious agents (2, 3). However, when ribozymes are used in vivo, not all of them were effective, most probably because of their low activity and/or instability in vivo. To overcome these problems, many approaches have been undertaken, for example, the chemical modifications and substitutions of nucleotides to improve the stability and activity of ribozymes.

As one of the methods for finding highly active ribozyme sequences, in vitro genetic selections have been used. The method takes advantage of a process that mimics evolution, namely mutation, amplification and selection. New functional ribozymes with ligase, kinase, amino-acid cleavage or selfalkylating activites have already been selected by this method. However, the drawback to selection in vitro is that the activity in vitro does not always reflect the activity in vivo.

Recently we reported a novel method for screening of active ribozymes using DHFR gene as a selective marker in E. Coli. (4-6). In this system, when cells were transformed with a mixture that consisted of active ribozyme-coding and inactive ribozyme-coding plasmids at a ratio of 1:1, it was mainly the cells expressing the active ribozyme that survived in the presence of TMP. Thus, this positive selection system in vivo was functional. However, the background "noise" could not be removed completely.

In this report, we show a novel in vivo selection system for active ribozymes in mammalian cells.

RESULTS AND DISCUSSION
To select active ribozymes in mammalian cells, we needed morphologically distinguishable changes, such as focus and colony formations, that would enable us to differentiate cells harboring active ribozymes from other cells that harbor inactive ribozymes. The focus or colony formation reflects one of phenotypes caused by transformation of cells. In our selection system, we chose Balb3T3 cell line, that originated from mouse fibroblast cells, as the host cell line. This is because the Balb3T3 cell line is very sensitive to genotoxic agents and oncogenes such as v-ras, v-src and v-myc, and is also sensitive to the presence or absence of
tumor suppressor gene product and, thus, this cell line is used for the analysis of ability of transformation of cells by those agents/factors. The cyclin dependent kinase inhibitor (CDKI) \( p16^{INK4a} \) is known as a tumor suppressor gene product which participates in the G1 arrest during the cell cycle event. Thus, we thought that if ribozymes could cleave \( p16^{INK4a} \) mRNA in Balb3T3 cells, as a result, those active ribozyme should initiate focus and colony formations.

In order to identify the best (close to the best) target site for the \( \text{trans-acting} \) ribozymes, we at first constructed eight kinds of \( \text{tRNA}^{Val} \)-driven ribozymes, each targeting separately to specific one of the eight different sites in \( p16^{INK4a} \) mRNA. Expression of these ribozymes is controlled by a human \( \text{tRNA}^{Val} \) promoter and is known as the pol III dependent expression system. This system is suitable for the expression of short RNAs and the level of expression is at least from 10 to 100-fold higher than that expressed by the pol II dependent expression system. Indeed, we also demonstrated the appropriateness of this expression system especially for ribozymes. Moreover, we can design the pol III based expression vector in such a way that these ribozyme transcripts be exported to the cytoplasm to ensure co-localization of the ribozyme with its target mRNA.

Balb3T3 fibroblast cells were transfected with these ribozyme expression plasmids. Cells expressing \( p16^{INK4a} \)-directed ribozymes were incubated for 2 to 3 weeks. Among eight kinds of ribozyme expressing cell type, ribozyme 1 (R1), ribozyme 2 (R2), ribozyme 3 (R3), ribozyme 4 (R4) and ribozyme 8 (R8) expressing cells produced foci as a result of transformation (Fig. 1). Since the ribozyme 3 expressing cells formed the largest number of foci, subsequent ribozymes were designed and targeted at R3 site. It should be mentioned that the vector encoding the \( \text{tRNA}^{Val} \) promoter alone did not initiate any focus formation.

To examine whether production of foci was caused by the reduction of \( p16^{INK4a} \) by the action of ribozymes, we checked the level of \( p16^{INK4a} \) mRNA using reverse transcription with polymerase chain reaction (RT-PCR) analysis. The level of \( p16^{INK4a} \) mRNA was drastically reduced in Balb3T3 expressing an active \( p16^{INK4a} \)-directed ribozyme compared with untreated Balb3T3 cells.

To test whether the reduction of \( p16^{INK4a} \) mRNA was caused by the cleavage by the ribozyme, we constructed inactive ribozyme expression plasmids which had a single mutation of G5 to A5 in the catalytic core. Balb3T3 cells expressing inactive ribozymes were selected by G418 resistance two to three weeks after the transfection. The inactive ribozyme did not alter mRNA levels of \( p16^{INK4a} \). Thus, these results clearly demonstrated that production of foci were caused by reduction of \( p16^{INK4a} \) gene products by \( p16^{INK4a} \)-directed active ribozymes.

Therefore, our \( \text{in vivo} \) selection system has a potential to design and identify hyper-active ribozymes. Our present analysis also demonstrates that the activity of ribozymes \( \text{in vivo} \) heavily depends on their target site (Fig. 1).

**REFERENCES**