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Transport of intracellularly active ribozymes to the cytoplasm

Abstract Ribozymes are RNA molecules with enzymatic activity which selectively bind and cleave specific target RNAs. To date, numerous studies directed toward the application of ribozymes in vivo have been performed and many successful experiments have been reported. However, to induce high-level activities of ribozymes in vivo, several factors must be considered. Here we report that the cytoplasmic localization of ribozymes is important for their intracellular activity in mammalian cells. Northern blot analysis revealed that a $tRNA^{Val}$ ribozyme, which can assume a cloverleaf structure similar to that of a native tRNA, is efficiently transported to the cytoplasm. In contrast, the $tRNA_i^{Met}$-driven ribozyme, which does not maintain the cloverleaf structure, remained predominantly in the nucleus. In correlation with the localization, the activity of the exported ribozyme was higher than that of the ribozyme retained in the nucleus. These results should provide insight into the design of ribozymes that have high-level activity in mammalian cells.

Keywords Ribozyme · Hammerhead ribozyme · Maxizyme · Export

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Introduction

Many successful attempts to use ribozymes for the suppression of gene expression have been reported [3, 4, 8, 10, 11, 12, 13, 14, 15, 16, 17, 19, 21, 24, 25, 26, 27, 29, 31, 33, 34, 40, 41]. However, the efficacy of ribozymes in vitro is not necessarily correlated with their functional activity in vivo [17]. When a ribozyme is generated within a cell by transcription from an expression vector, factors such as the transcription rate, stability and localization of the transcript, and cleavage activity are likely to influence the functional activity of the ribozyme in vivo [2, 7, 17, 37]. Since such factors depend in large part on the activities of cellular proteins, it is important that the characteristics of a ribozyme be appropriate for optimal functioning in the intracellular environment. Thus the successful intracellular action of a ribozyme depends significantly on the choice of expression system.

At present, the RNA polymerase system II (pol II system) and RNA polymerase system III (pol III system) are the main systems used for the expression of ribozymes in eukaryotic studies. In our studies, pol III-driven ribozymes [17, 19, 20, 21], but not pol II-driven ribozymes [28] were detected by Northern blot analysis, providing evidence of the higher expression level of the former transcripts. Thus the pol III system appears to be particularly useful for the expression of ribozymes. In addition, in the case of a tRNA promoter, since each expression unit is compact (fewer than 200 bp including the ribozyme sequence), the promoters are suitable for the expression of multiple ribozymes (tRNA-Rzs) from a single vector, such as a retroviral vector. However, in many cases, the expected effects of ribozymes have not been achieved, despite the apparently favorable features of the pol III system.

In this study, we investigated the correlation between the intracellular localization and activity of various tRNA-Rzs in mammalian cells. The results indicate that the cytoplasmic localization of the tRNA-Rz is critical for its high-level intracellular activity.

Materials and methods

Construction of plasmids for expression of tRNA-Rz

The construction of ribozyme expression vectors using pUC-dt (a plasmid that contains the chemically synthesized promoter for a human gene for $tRNA^{Val}$ between the *Eco*RI and *Sal* I sites of pUC 19) has been described previously [17, 19, 20]. In all cases, the pUC-dt was double-digested by *Csp* 45I and *Pst* I and each ribozyme sequence, with the terminator sequence UUUUU at the 3′ end, was ligated into the plasmid.

Northern blot analysis

Cells were grown to approximately 80% confluence (1×10^7 cells) and transfected with a tRNA-Rz expression vector with the Lipofectin reagent (Gibco-BRL, Gaithersburg, Md.). Cells were harvested 36 h after transfection. For the preparation of the cytoplasmic fraction, collected cells were washed twice with phosphate-buffered saline (PBS) and then resuspended in digitonin lysis buffer (HEPES 50 mM/KOH, pH 7.5, potassium acetate 50 m*M*, $MgCl_2$ 2 mM, EGTA 2 mM, and digitonin 50 μg/ml) on ice for 10 min. The lysate was centrifuged at 1000 *g* and the supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in NP-40 lysis buffer (Tris-HCl 20 mM, pH 7.5, KCl 50 mM, NaCl 10 mM, EDTA 1 mM, and 0.5% NP-40), held on ice for 10 min, and the resultant lysate was used as the nuclear fraction. Cytoplasmic RNA and nuclear RNA were extracted and purified from the fractions with ISOGEN reagent (Wako, Osaka, Japan). Total RNA (30 μg per lane) were loaded on a 3.0% NuSieve (3:1) agarose gel (FMC, Rockland, Me.). After electrophoresis, bands of RNAs were transferred to a Hybond-N nylon membrane (Amersham, Little Chalfont, UK). The membrane was probed with synthetic oligonucleotides complementary to the sequences of the relevant ribozymes, each labeled with ^{32}P using T4 polynucleotide kinase (Takara Shuzo Company, Kyoto, Japan).

Luciferase assay

HeLa cells were plated at 80% confluence in 12-well plates and incubated at 37°C in a CO_2-enriched atmosphere. The cells were washed twice with PBS before cotransfection. Ribozyme expression plasmids (3 μg of each) and 500 ng of the target gene-expressing plasmid, which encoded the chimeric HIV-1 LTR sequence-luciferase gene (pGV-V1), were mixed with lipofectin reagent 4 μl in 400 μl of serum-reduced medium (OPTI-MEM I; Gibco-BRL) and incubated for 30 min at room temperature as described in the manufacturer's instructions. The mixture was then gently added to cells. After 12 h, the medium was replaced with growth medium (Dulbecco's minimum essential medium, DMEM) supplemented with 10% fetal calf serum, and cells were cultured for a further 24 h.

Luciferase activity was measured with a PicaGene kit (Toyo-inki, Tokyo, Japan) as described elsewhere [17, 20]. To normalize the efficiency of transfection by reference to β-galactosidase activity, cells were cotransfected with pSV-β-galactosidase control vector (Promega, Madison, Wis.), and the chemiluminescent signal due to β-galactosidase was determined with a luminescent β-galactosidase genetic reporter system (Clontech, Palo Alto, Calif.) as described previously [17].

Results

In studies directed toward applications of hammerhead ribozymes in vivo [7, 18, 22, 23, 30, 32, 36, 38, 39], a tRNA-based system for expression of ribozymes was established which resulted in both high-level expression and intracellular stability [3, 5, 6, 40]. In addition, it appears that colocalization of a ribozyme with its target is an important determinant of ribozyme efficiency [3, 6, 9, 13, 14, 15, 17, 19, 20, 21, 33]. Our tRNA-attached ribozymes ($tRNA^{Val}$-Rz; Fig. 1) with extremely high activity in mammalian cells [1, 13, 15, 17, 19, 20, 35] can be classified as nonaminoacylated immature tRNAs because each has an unprocessed 5′ end and an extended 3′ end (which includes the ribozyme sequence) without a

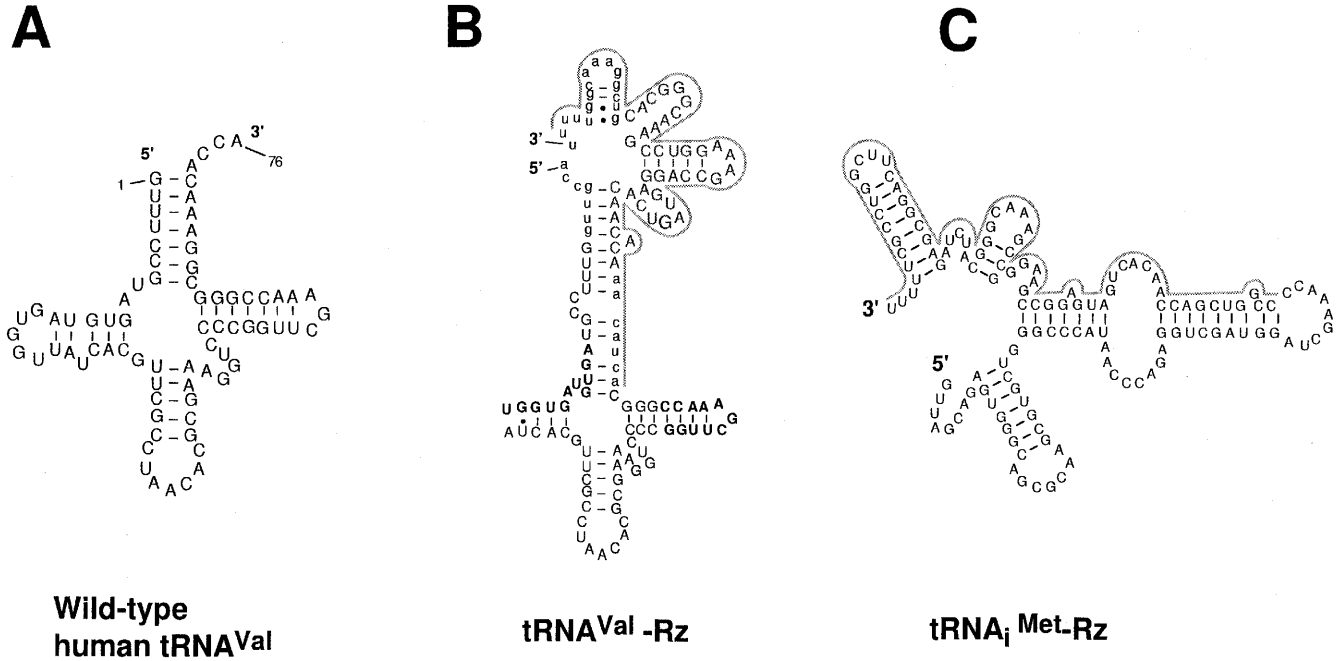


Fig. 1A–C Secondary structures of wild-type $tRNA^{Val}$ (**A**), $tRNA^{Val}$-Rz (**B**), and $tRNA_i^{Met}$-Rz (**C**). The sequences underlined are complementary to the sequences of probes used for Northern blot hybridization

3′ CCA end. In our RNA pol III transcription system, the promoter is located within the tRNA sequence being transcribed. Therefore it is inevitable that a portion of the tRNA becomes incorporated into the ribozyme. In our expression system, the ribozymes are linked downstream from the partially modified human $tRNA^{Val}$ through a linker (Fig. 1B).

To examine the subcellular localization of various types of tRNA-driven ribozyme, we assayed Northern blots using nuclear and cytoplasmic total RNA. HeLa S3 cells were grown to approximately 80% confluence (1×10^7 cells) and transfected with the vector that encoded the $tRNA^{Val}$-Rz (Fig. 1B) or $tRNA_i^{Met}$-Rz (Fig. 1C). Total RNAs were separated into nuclear and cytoplasmic fractions 36 h after transfection and analyzed by hybridization. Transcripts of approximately 130 nucleotides in length, corresponding in size to $tRNA^{Val}$-Rz, were detected only in the cytoplasm (Fig. 2A). In contrast, $tRNA_i^{Met}$-Rz and the control U6-driven ribozyme were preferentially retained in the nucleus (Fig. 2B).

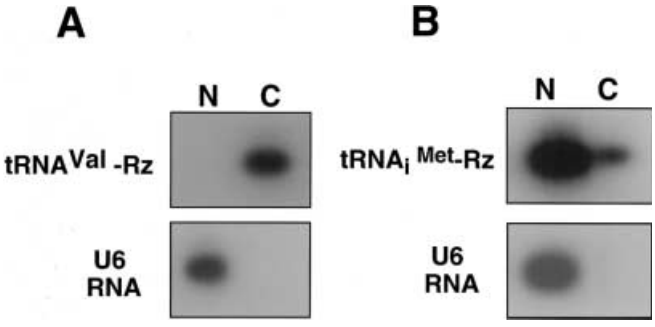


Fig. 2A, B Steady-state levels of expression and intracellular localization of $tRNA^{Val}$-Rz (**A**), and $tRNA_i^{Met}$-Rz (**B**). Northern blot analysis was performed with total RNA from intracellular fractions (*N* nuclear, *C* cytoplasmic)

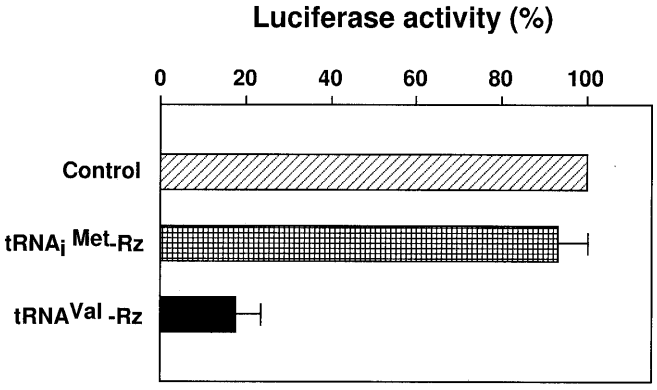
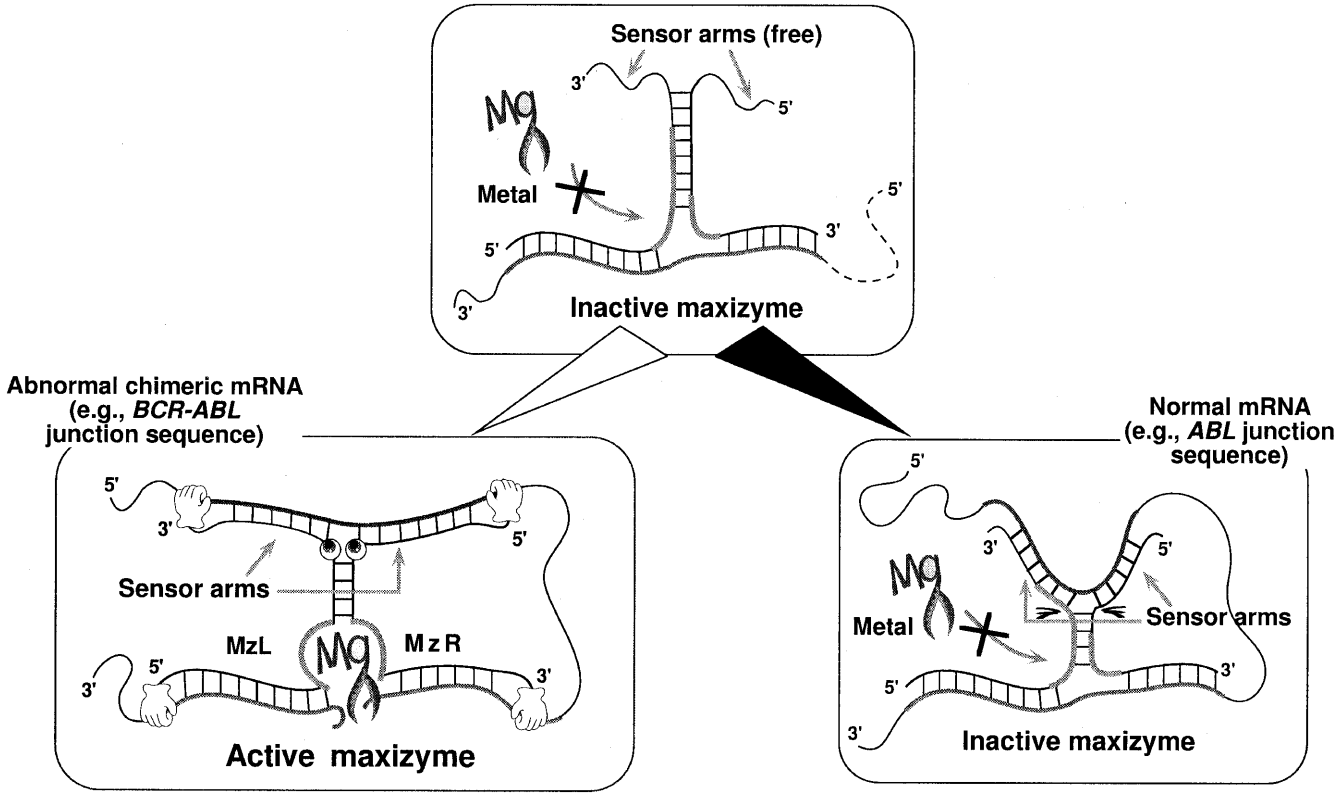


Fig. 3 Comparison of intracellular activities of the nuclear- and cytoplasmic-localizing ribozyme in HeLa cells. The results shown are the averages of results from four sets of experiments

Fig. 4 Formation of an active or an inactive maxizyme by dimerization, which is regulated allosterically by a specific effector sequence. The heterodimer (*MzL* and *MzR*) can generate two different binding sites, one complementary to the sequence of interest (*sensor arms*), and the other complementary to a cleavable sequence



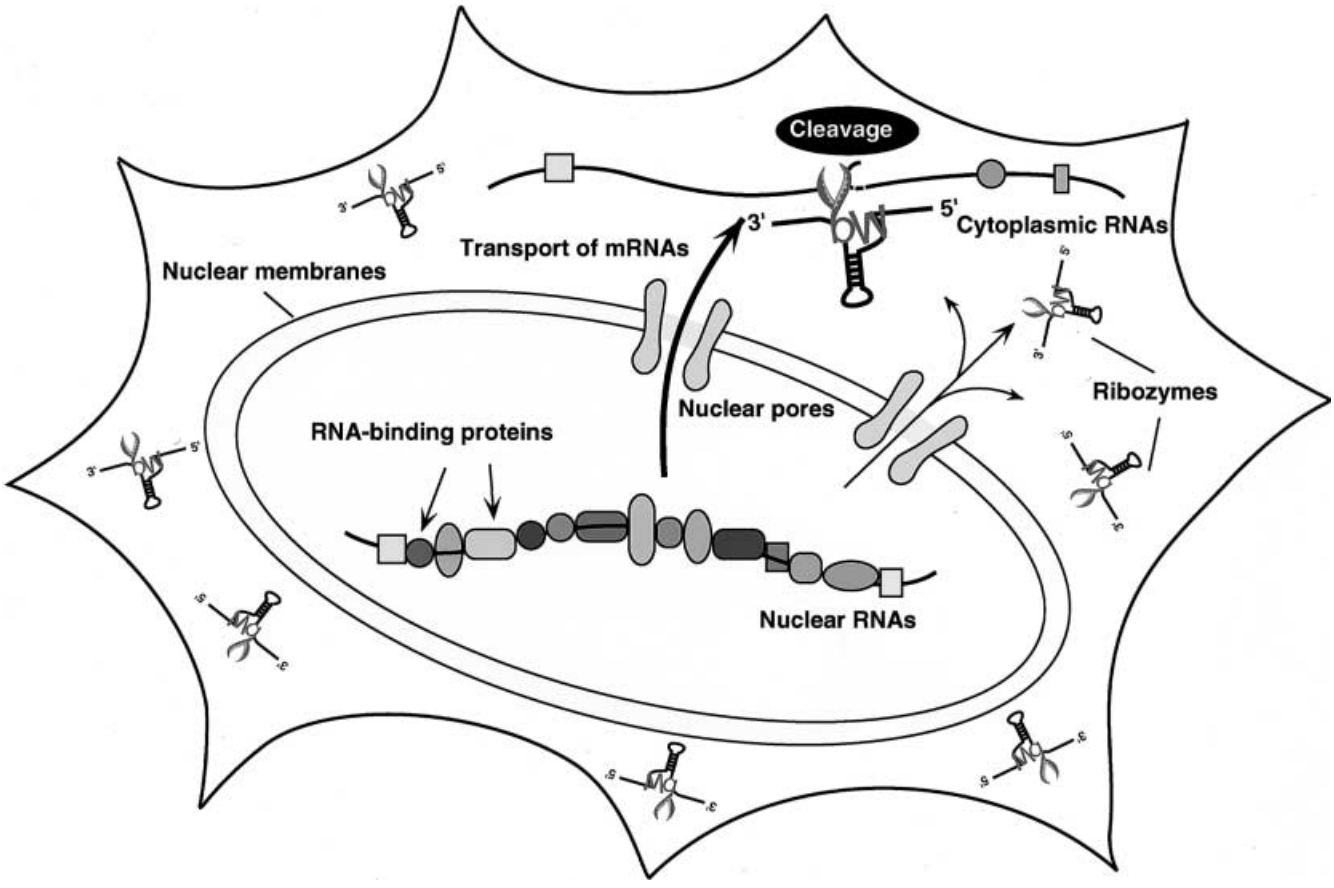


Fig. 5 Export of transcribed ribozymes. Cytoplasmic ribozymes appear significantly more active than nuclear ribozymes, suggesting that mature mRNAs in the cytoplasm might be more accessible to antisense molecules than are pre-mRNAs in the nucleus

Computer analysis of RNA secondary structures predicted that the transported $tRNA^{Val}$-Rz, as well as other intracellularly active ribozymes, maintained cloverleaf structures similar to that of a native tRNA (Fig. 1B, and data not shown), whereas $tRNA_i^{Met}$-Rz and other transcripts that remained predominantly in the nucleus had structures different from that of a tRNA (Fig. 1C, and data not shown). Thus it appears that, in the case of the export of tRNA ribozymes with extended 5′ and 3′ ends (tRNA-Rz), receptors involved in the recognition of native tRNA might also be responsible for the export of tRNA-Rz in mammalian cells.

We next compared the catalytic activities of the cytoplasmic and nuclear localizing ribozymes in mammalian cells. Although $tRNA^{Val}$-Rz and $tRNA_i^{Met}$-Rz had the identical ribozyme sequence targeting to an identical site on HIV-1 LTR mRNA, they had different localizations (Fig. 2) and secondary structures (Fig. 1). This was due to the different linker sequence that was inserted between the tRNA and ribozyme sequences, as shown in Fig. 1. Each tRNA-Rz expression plasmid was transfected into HeLa cells along with a target gene-expressing plasmid, which encoded a chimeric target HIV-1 LTR sequence-luciferase gene. As shown in Fig. 3, despite the fact that $tRNA^{Val}$-Rz and $tRNA_i^{Met}$-Rz had similar expression levels in mammalian cells (Fig. 2), the intercellular activity of the ribozyme transported into the cytoplasm ($tRNA^{Val}$-Rz) was significantly higher than that of the ribozyme remaining in the nucleus (Fig. 3).

Discussion

A ribozyme is a potentially useful tool for the suppression of the expression of specific genes since it can be engineered to act on other RNA molecules with high specificity. Although many trials have been successful, it remains difficult to design an effective ribozyme expression system that can be used in vivo. One major challenge related to the use of ribozymes and antisense RNAs as therapeutic or genetic agents is the development of suitable expression vectors. We used the pol III system and the promoter of a human gene for $tRNA^{Val}$ for transcription of ribozymes. This promoter is not only suitable for transcription of small RNAs, but its use also facilitates prediction of secondary structure by computer folding. More importantly, if properly designed, it allows the export of transcribed $tRNA^{Val}$-Rzs from the nucleus to the cytoplasm so that the $tRNA^{Val}$-Rzs can find their mRNA targets. The $tRNA^{Val}$ vector may be useful for expression of functional RNAs other than ribozymes for which the target molecules are localized in the cytoplasm. Although colocalization in the cytoplasm cannot in itself guarantee effectiveness, we can clearly increase the

probability of success. In our studies, $tRNA^{Val}$-Rzs had consistently high activity, at least in cultured cells.

It should be emphasized that the maxizyme, an allosterically controllable ribozyme with powerful biosensor capacity (Fig. 4) which appears to function even in mice [18, 20, 22, 23, 35, 39], was also driven under the control of the $tRNA^{Val}$ promoter. The biosensor functions of the maxizyme allow specific inhibition of expression of the gene of interest, with no effect on normal mRNA (Fig. 4). The maxizyme provides the first example of successful allosteric control of the activity of an artificially created allosteric enzyme, not only in vitro but also in animals, and its potential utility in medicine cannot be ignored. By modulating the sequences of sensor arms, we can easily adjust the activity of the maxizyme. Thus maxizymes are powerful gene-inactivating agents with allosteric functions which can cleave any type of chimeric mRNA and/or any RNA by recognizing a specific sequence of interest for cancer gene therapy. We have confirmed that the transcripts must be localized in the cytoplasm to maintain the activity of the ribozyme/maxizyme in cells (Fig. 5).

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