Relationships between the Activities in Vitro and in Vivo of Various Kinds of Ribozyme and Their Intracellular Localization in Mammalian Cells*

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Yoshio Kato‡§¶, Tomoko Kuwabara¶¶, Masaki Warashina§§¶, Hirofumi Toda§, and Kazunari Taira¶¶**

From the ‡Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, 7-3-1 Hongo, Tokyo 113-8656 and the §Gene Discovery Research Center, National Institute of Advanced Industrial Science and Technology, 1-1-4 Higashi, Tsukuba Science City 305-8562, Japan

Nineteen different functional RNAs were synthesized for an investigation of the actions of ribozymes, in vitro and in vivo, under the control of two different promoters, tRNA or U6, which localize transcripts either in the cytoplasm or in the nucleus. No relationships were found between the activities of these RNAs in cultured cells and the kinetic parameters of their respective chemical cleavage reactions in vitro, indicating that in no case was chemical cleavage the rate-limiting step in vivo. For example, a hepatitis delta virus (HDV) ribozyme, whose activity in vitro was almost 3 orders of magnitude lower than that of a hammerhead ribozyme, still exhibited similar activity in cells when an appropriate expression system was used. As expected, external guide sequences, the actions of which depend on nuclear RNase P, were more active in the nucleus. Analysis of data obtained with cultured cells clearly demonstrated that the cytoplasmic ribozymes were significantly more active than the nuclear ribozymes, suggesting that mature mRNAs in the cytoplasm might be more accessible to antisense molecules than are pre-mRNAs in the nucleus. Our findings should be useful for the future design of intracellularly active functional molecules.

Since the discovery of the first two ribozymes (1, 2), several new types of ribozyme with self-cleavage activity have been found in nature (3–8). Small ribozymes that can be designed to cleave RNA strands intramolecularly include hammerhead, hairpin, and HDV1 ribozymes. These trans-acting ribozymes recognize their RNA substrates via formation of Watson-Crick base pairs, and they cleave these RNAs in a sequence-specific manner. Because of their specificity, trans-acting ribozymes show promise as tools for the dysfunction of target RNAs (9–21).

The constitutive expression of a ribozyme in vivo, under the control of a strong promoter, represents an attractive strategy for the application of trans-acting ribozymes to gene therapy. As described in our previous reports (22, 23), we have succeeded in establishing an effective ribozyme expression system, with subsequent efficient transport of transcripts to the cytoplasm, which is based on a promoter that is recognized by RNA polymerase III (pol III). High levels of expression under the control of the pol III promoter are advantageous for the exploitation of ribozymes in vivo. Therefore, we chose an expression system with the promoter of a human gene for tRNAVal. Many ribozymes, such as hammerheads and hairpins, have been effectively expressed under the control of promoters of gene for tRNAs (9, 11–15, 20, 21, 24).

A major advantage of our tRNAval-directed expression system is that, with appropriate modification of the tRNAval portion, it is possible to colocalize the expressed ribozyme in the cytoplasm with its target mRNA (14, 15, 22, 23, 25). Ribozymes expressed under the control of the tRNAval promoter are exported to the cytoplasm as effectively as natural tRNAs via the action of Xpo(t),2 a tRNA-binding protein (26, 27) that functions with Ran GTPase, which regulates the transport by catalyzing the hydrolysis of GTP. Mature mRNAs are exported to the cytoplasm for translation. Thus, both ribozymes and their target mRNAs can be co-localized in the same cellular compartment.

By contrast, an external guide sequence (EGS), which is added in trans and is able to bind to its target RNA, appears to function in the nucleus because its effect depends on the activity of ribonuclease P (RNase P) (17, 28–30). The EGS RNA binds to the target RNA, yielding a structure that resembles the pre-tRNA that is recognized as a substrate by RNase P. RNase P normally cleaves precursors to tRNAs to generate the 5′ termini of mature tRNAs. Because RNase P is expressed constitutively in cells and accumulates, in particular in the nucleus, the use of an EGS as a gene-inactivating agent does not require expression of additional RNase P from exogenously introduced genes. Although an EGS does not have intrinsic cleavage activity, when it acts in cooperation with endogenous RNase P, it can effectively inactivate its target mRNA.

Although there have been many studies both in vitro and in vivo of the activities of the ribozymes mentioned above, further detailed information on the parameters that determine their activities as gene-inactivating agents in vivo is necessary so that we will be able to optimize their effects by optimizing the

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† These authors contributed equally to this work.

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§ To whom correspondence should be addressed: Dept. of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, Hongo, Tokyo 113-8656, Japan. Tel.: 81-3-5841-8828 or 81-298-61-3015; Fax: 81-298-61-3019; E-mail: taira@chembio.t.u-tokyo.ac.jp.

1 The abbreviations used are: HDV, hepatitis delta virus; pol, polymerase; EGS, external guide sequence; CML, chronic myelogenous leukemia; nt, nucleotide(s); Wt, wild-type; Rz, ribozyme.

2 T. Kuwabara and K. Taira, unpublished data.
requisite parameters. In addition, although it has been claimed for each individual ribozyme that it has potential utility as an effective gene-inactivating agent, there has been no systematic analysis in which the activities of various ribozymes have been compared under similar conditions in vitro. In this study, we designed several types of functional RNA targeted to the junction site of the BCR-ABL chimeric mRNA that causes chronic myelogenous leukemia (CML). Using this system, we have accumulated data that might allow correlations to be made between ribozyme activities in cultured cells and the efficacies of the same ribozymes in vivo, namely, in mice (15, 21). CML occurs as a result of reciprocal chromosomal translocations that result in the formation of the BCR-ABL fusion gene. One of the chimeric mRNAs transcribed from an abnormal BCR-ABL (B2A2) gene (consisting of exon 2 of BCR and exon 2 of ABL; Refs. 31 and 32) provides a suitable substrate for comparisons of ribozymes. We used six kinds of functional RNA, including hammerhead, hairpin, and HDV ribozymes; our maxizyme and in vitro selected minizymes; and EGSs to examine parameters that determined activities in vitro and in vivo.

Our goal was to determine whether activity in vitro might reflect activity in mammalian cells. Moreover, since we have evidence that suggests that tRNA<sup>val</sup>-driven ribozymes with high level of activities are efficiently exported to the cytoplasm, whereas similarly expressed tRNA ribozymes with poor activities are accumulated in the nucleus (22), we decided to examine the correlation between nuclear localization and/or transport of functional RNAs and the activity in vitro. For this purpose, we used two kinds of promoter. One promoter was the promoter of the gene for tRNA<sup>val</sup> described above, and the other was a U6 promoter (33, 34). Transcripts expressed under the control of these promoters are located in the cytoplasm and the nucleus, respectively.

We found that the intrinsic cleavage activity of a ribozyme is not the sole determinant of activity in cultured cells and that it is the cytoplasmic localization and the association of the ribozyme with its substrate that regulate activity.

MATERIALS AND METHODS

Construction of Vectors for Expression of Ribozymes and EGSs—The construction of vectors for expression of ribozymes from the tRNA<sup>val</sup> promoter using pUC-dt (a plasmid that contains the chemically synthesized promoter for a human gene for tRNA<sup>val</sup> between the EcoRI and SalI sites of pUC 19) was described previously (22, 23). pUC-dt was double-digested with Csp<sub>45I</sub> and SalI, and a fragment having a linker sequence with 5' Csp<sub>45I</sub> site and the restriction sites for KpnI and EcoRV and the terminator sequence TTTTT at the 3' end with 3' SalI site was cloned into the double-digested plasmid to yield pUC-tRNA/KE. The KpnI and EcoRV sites were used for subsequent insertion of the each ribozyme sequence. The construction of vectors for ribozyme expression from the U6 promoter has been described elsewhere (17). The EcoRI and XhoI sites were used for insertion of each ribozyme sequence.

Analysis of the Cleavage Activity of Individual Ribozymes in Vivo—Each ribozyme and two substrates, namely BCR-ABL and ABL RNAs, were prepared in vitro using T7 RNA polymerase. Assays of ribozyme activity in vitro were performed in 25 mM MgCl<sub>2</sub> and 50 mM Tris-HCl (pH 8.0) at 37 °C under enzyme-saturating (single-turnover) conditions, as described elsewhere (14). Each ribozyme (50 μM) was incubated with 2 μM 5'-<sup>32</sup>P-labeled substrate. The substrate and the products of each reaction were separated by electrophoresis on an 8% polyacrylamide, 7% urea denaturing gel and detected by autoradiography.

In Situ Hybridization—HeLa S3 cells on a coverslip, which had been transfected in advance with an appropriate plasmid, were washed in fresh phosphate-buffered saline and fixed in 2% peroxidization buffer (50 mM HEPES/KOH, pH 7.5, 50 mM potassium acetate, 8 mM MgCl<sub>2</sub>, 2 mM EGTA, and 50 μg/ml digitonin) on ice for 10 min. The lysate was centrifuged at 1,000 × g, and the supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) and held on ice for 10 min, and the resultant lysate was used as the nuclear fraction. Cytoplasmic RNA and nuclear RNA were extracted from the cytoplasmic and nuclear fraction, respectively, with ISOGEN reagent (Wako, Osaka, Japan). Thirty micrograms of total RNA per lane were loaded on a 3.0% NuSieve<sup>®</sup> (3:1) agarose gel (FMC Inc., Rockland, ME). After electrophoresis, bands of RNA were transferred to a Hybond-N<sup>®</sup> nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The membrane was probed with a synthetic oligonucleotide that was complementary to the sequence of the relevant ribozyme. Each probe was labeled with 32<sup>P</sup> by T4 polynucleotide kinase (Takara Shuzo Co., Kyoto, Japan).

Measurement of Luciferase Activity—Luciferase activity was measured by a PicoGene<sup>®</sup> kit (Toyo-inki, Tokyo, Japan) as described elsewhere (15). In order to normalize the efficiency of transfection by reference to ß-galactosidase activity, cells were cotransfected with the pSV-ß-galactosidase control vector (Promega, Madison, WI), and then the chloromineuxis signal due to ß-galactosidase was quantitated with a luminonex ß-galactosidase genetic reporter system (CLONTECH, Palo Alto, CA) as described previously (15).

RESULTS

Design of Ribozymes and EGSs—In order to express ribozymes in vitro, we used two kinds of pol III promoter (Fig. Ia). Transcripts with the promoter of the gene for tRNA<sup>val</sup> can be efficiently transported to the cytoplasm when the appropriate choice of and combination of linker and ribozyme sequence is made (14, 15, 22, 23, 25). We used the mouse U6 promoter, which controls expression of U6 RNA that is localized in the nucleus, for expression and accumulation of transcripts in the nucleus. Ten functional RNAs (Fig. 1b) directed against sites within a limited region (~100 nt) of B2A2 and ABL mRNAs (Fig. 1c; target sites are underlined; the identical cleavage site could not be chosen because of different cleavable sequence for each different ribozyme) and expression vectors that encoded each respective functional RNA were designed such that each functional RNA was produced under the control both of the tRNA<sup>val</sup> promoter and of the U6 promoter. The product translated from B2A2 chimeric mRNA causes CML. We demonstrated previously that the RNA maxizyme that functions as a dimer cleaves B2A2 chimeric mRNA in vitro and in vivo with extremely high specificity without any damage to normal ABL mRNA (15, 21). Since it seemed possible that a hammer ribozyme might also distinguish abnormal B2A2 mRNA from normal ABL mRNA in vivo, we decided to use these two substrates in this study. Using two different substrates, we hoped to gain more insight into the differences among the activities of the various ribozymes in vivo in more general terms.

The hammerhead ribozyme is one of the smallest transacting ribozymes (6, 7, 19, 35–37). The stem II region of this...
Cleavage Activities of Ribozymes in vitro and in vivo

Comparison of Ribozymes in Vitro and in Vivo

We first determined activities of the various ribozymes in vitro. In order to compare chemical cleavage activities rather than association and/or dissociation kinetics, we measured the activities of the functional RNAs in vitro in the presence of a saturating excess of each ribozyme (single-turnover conditions). Ribozymes and substrate RNAs were transcribed in vitro by T7 RNA polymerase. As substrates, we used RNAs of 92 and 121 nt, which corresponded to regions that spanned the junctions of ABL RNA and B2A2 RNA, respectively (Fig. 1C). Because the tRNAVal promoter is an internal promoter, in other words the DNA sequence that corresponds to tRNAVal contains the promoter region, transcripts from this promoter are always linked to a portion of the RNA, which might or might not interfere with the ribozyme’s activity. Therefore, ribozymes tested in vitro included a modified tRNA promoter region (about 90 nt) or about 20 nt of the U6 promoter (Fig. 1A).

As shown in Fig. 2, wild-type hammerhead ribozymes (Wt Rz) had the highest activity in the case of both the tRNAVal and U6 promoter-driven transcripts and against both the B2A2 and ABL substrates. Since the majority of the target sites are located in the exon 2 region of ABL RNA and since the computer-predicted secondary structure of this region is almost the same for both substrates (Fig. 1C, dark blue), with the exception of the region upstream of the junction, no significant differences between the rates of cleavage by the ribozymes of B2A2 and ABL substrates were expected or observed. In general, rates of cleavage by tRNAVal-driven ribozymes were slightly higher than those by U6-driven ribozymes, demonstrating that the tRNA portion did not hinder the activity of the hammerhead ribozyme; the activity of the majority of ribozymes against the relatively long substrates (92 and 121 nt) was 2 or more orders of magnitude lower than that of Wt Rz in vitro.

Localization of tRNAVal- and U6-driven Transcripts—Colocalization of a ribozyme with its substrate is an important determinant of the activity of the ribozyme (10, 15, 22, 23). A transcribed ribozyme might be expected to cleave pre-mRNAs in the nucleus or to be exported to the cytoplasm to cleave mature mRNAs (19). Our earlier data indicate that tRNAVal-driven ribozymes with high level of activities are efficiently exported to the cytoplasm, while similarly expressed tRNAribozymes with low level of activities are accumulated in the nucleus (22). However, there has been no systematic attempt to identify the cellular compartment in which a ribozyme acts most effectively. Given that it should be necessary for a ribozyme to be transported to the cytoplasm in mammalian cells for colocalization with its target mRNA, we developed our expression system for cytoplasmic expression of ribozymes, because mature mRNAs are exported to the cytoplasm for translation. By contrast, an EGS is likely to be operative in the nucleus because cleavage depends on RNase P, which is active only in the nucleus. In order to confirm this hypothesis (see next section), ribozymes were expressed from both types of promoter and their localization was determined both by in situ hybridization and by Northern blotting analysis of fractionated cell lysates.
HeLa cells were transfected with plasmids with a tRNA\textsuperscript{Val} or U6 promoter. After 36 h, we examined the localization of each expressed ribozyme by \textit{in situ} hybridization and Northern blotting analysis of fractionated cells. For \textit{in situ} hybridization, Cy3-labeled probes were incubated with fixed and permeabilized cells and then the fluorescence of Cy3 was detected by confocal microscopy. In this way, we confirmed the expected locations of all 19 different transcripts. Typical examples of our results are shown in Fig. 3, in which the red signals indicate the presence of a hairpin ribozyme (top) or an EGS (bottom). In all cases examined, without exception, tRNA\textsuperscript{Val}-driven transcripts were transported to the cytoplasm and U6-driven ribozymes and U6-driven ribozymes were measured and the results are summarized in C and D, respectively. Mini, mini-enzyme; Hair, hairpin.

For Northern blotting analysis, HeLa cells were fractionated to yield nuclear and cytoplasmic fractions and RNA was extracted from each fraction. This RNA was allowed to hybridize with an appropriate \textsuperscript{32}P-labeled probe after electrophoresis (Fig. 4). Without exception, tRNA\textsuperscript{Val}-driven ribozymes and EGSs expressed under control of the tRNA\textsuperscript{Val} promoter were found in the cytoplasmic fraction and U6-driven transcripts were found in the nuclear fraction. The levels of all transcripts were very similar (they differed by less than 20%), irrespective of the type of ribozyme expressed and the expression system (tRNA\textsuperscript{Val} or U6 promoter). Since the steady-state level of the transcript (reflecting its stability in cells) is a major determinant of ribozyme activity \textit{in vivo}, if levels of transcripts had not been similar, our comparison of activities \textit{in vivo} would have been more difficult (see the next section).

The Activities of Various Functional RNAs in Cultured Cells—We cotransfected HeLa cells with an expression plasmid that encoded an appropriate ribozyme unit(s) under the control of the tRNA\textsuperscript{Val} or U6 promoter, and a plasmid that encoded the target \textit{BCR-ABL} or ABL sequence fused with a gene for luciferase (\textit{luc}), to evaluate the intracellular activity of ribozymes. The plasmid pB2A2-luc contained a sequence of B2A2 mRNA, while pABL-luc contained a sequence of 300 nt that encompassed the same target cleavage site and the junction between exon 1 and exon 2 of normal ABL mRNA. After transient expression of the ribozyme, substrate, and luciferase in individual cell lysates, we estimated the intracellular activity of each ribozyme by measuring luciferase activity. Our results are shown in Fig. 5. The luciferase activity recorded when we used each target gene-expressing plasmid (pB2A2-luc or pABL-luc) was taken as 100%. The data presented are the results of three to six independent experiments. However, the various sets of experiments were performed on different days and transfection efficiencies varied, depending on the conditions of cells on each specific day. As a consequence, standard errors (error bars) were relatively large. However, when experiments were carried out on the same day, standard
errors were in the range of 10–20%. Nonetheless, the rank order of the activities of ribozymes always remained the same; thus, the data presented in Fig. 5 can be compared at least qualitatively.

Expression of the tRNAVal portion by itself had no inhibitory effect. In all the cases when tRNAVal-driven ribozymes were directed against B2A2 target (results indicated by purple colors in Fig. 5) and ABL target (indicated by blue colors), the luciferase activity decreased (the U6-driven maxizyme was not constructed in this study). As expected and in accord with previous findings (15, 20, 21, 25, 45–47), the tRNAVal-driven maxizyme showed high level specificity, cleaving only B2A2 mRNA without damaging ABL mRNA (Fig. 5A). No other ribozyme was constructed in this study.

**Fig. 3.** Confocal microscopic images showing the detection by in situ hybridization of ribozymes and EGS expressed in mammalian cells. Cy3-labeled probes were used for detection of the tRNA Val-driven and U6-driven hairpin ribozymes. Similar images were obtained for all 19 different constructs, i.e. tRNA-driven ribozymes and EGSs were transported to the cytoplasm, and U6-driven ribozymes and EGSs were localized in the nucleus.

**Fig. 4.** Nuclear localization of functional RNAs. The steady-state levels of tRNAVal-driven ribozymes (A) and U6-driven ribozymes (B) and their localization are shown. Approximately the same levels of expression of functional RNAs from both promoters were observed. N, nuclear fraction; C, cytoplasm fraction. Without exception, tRNAVal-driven ribozymes and EGSs were localized in the cytoplasm and U6-driven ribozymes and EGSs were localized in the nucleus.

**Fig. 5.** Inhibitory effects in cultured cells of tRNAVal-driven ribozymes (A) and U6-driven ribozymes (B) on the expression of chimeric BCR-ABL-luciferase and ABL-luciferase genes. A plasmid that encoded a ribozyme or EGS and a plasmid that encoded the target gene were used to cotransfect HeLa cells. Decreases in luciferase activity (61) indicate the cleavage of transcripts by ribozymes in cells. The effects on the two different substrates are indicated by different colors. Mini, minizyme; Hair, hairpin.
able to distinguish between these two substrates. The extent of the decrease in luciferase activity was almost the same when in vitro selected minizymes were tested; they were slightly less effective than other ribozymes in cultured cells. As expected, tRNAVal-driven EGSs were ineffective since they were exported to the cytoplasm and their intracellular actions are known to depend on nuclear RNase P (Fig. 5A). We had expected that in vitro selected minizymes might be more active than their parental ribozymes because the minizymes were selected for the ability to act at low concentrations of Mg2+ ions (43). However, our expectations were not confirmed in cultured cells. Importantly, our data in cells demonstrate that those ribozymes, whose activity in vitro was almost 3 orders of magnitude lower than that of a hammerhead ribozyme, still exhibited significant activity in cells when an appropriate, high level expression system, which allows transport of ribozyme transcripts to the cytoplasm, was used.

When EGSs were expressed under control of the U6 promoter, they did demonstrate intracellular activity (Fig. 5B). The various other U6-driven ribozymes did not have any significant negative effects on expression of the luciferase gene. These results demonstrated clearly that only exported ribozymes (EGSs are not ribozymes per se) have significant cleavage activity because of the requirement for their colocalization with the target mRNA in the cytoplasm. Our data demonstrate that the target mRNAs in the cytoplasm are significantly more accessible to ribozymes than are the corresponding nuclear pre-mRNAs.

**DISCUSSION**

**Kinetics of Reactions in Vitro Reveal the Superior Cleavage Activity of a Hammerhead Ribozyme against Relatively Long Substrates**—To investigate the actions of various ribozymes, we first determined kinetic parameters in vitro under single-turnover conditions. The rate constants, kobs, which are summarized in Fig. 2 (C and D) and were obtained in the presence of excess ribozyme, reflected the rate of the chemical cleavage step because almost all of each substrate had been captured by each ribozyme when reactions were started by the addition of Mg2+ ions to the pre-heated and cooled ribozyme-substrate mixtures. Thus, the rate of the association step, which is a second-order reaction and was low under these conditions, could be ignored. In our studies, we used two relatively long substrates (92 and 121 nt) because kinetic parameters for short substrates have been well established and our purpose was to compare the activities of various ribozymes in vitro and in vivo against structured RNA substrates.

Fig. 2 shows that the activity of the hammerhead ribozyme (Wt Rz) was significantly higher than that of all the other ribozymes. This was true for both substrates and for two different transcripts (tRNAVal and U6-driven). The activity against the structured long substrate of the hammerhead ribozyme was about 2 orders of magnitude higher than that of the most of the other ribozymes. Nevertheless, the absolute activity, with the rate constants of 0.01–0.02 min−1 for the cleavage by the hammerhead ribozyme of the long substrate, was 2 orders of magnitude lower than the absolute activity against a short substrate. Under similar conditions, short RNA substrates can be cleaved by hammerhead ribozymes with rate constants of 1–2 min−1. The difference reflects the fact that longer RNA substrates tend to form structures that limit access by ribozymes (60). With our long substrates we showed that the hammerhead ribozyme was the best ribozyme for cleavage of such structured RNAs despite the fact that the hairpin ribozyme can cleave short substrates as efficiently as hammerhead ribozymes, with rate constants of 1–2 min−1 (24).

**Absence of Any Correlation between the Activities of Ribozymes in Vitro and in Cultured Cells**—Each functional RNA exhibited cleavage activity both in vitro and in cultured cells but with varying efficiency. As expected, the hammerhead ribozyme had significant activity in cultured cells (Fig. 5). We did not expect 100% inhibition in our transient expression assays because not all cells would have been transfected by the various respective plasmids. As seen from Fig. 5, there was no correlation between the trends in the ribozyme activity in vitro (Fig. 2) and in those in cultured cells (Fig. 5). The kinetic parameters obtained in vitro indicated that the hammerhead ribozyme was superior to other ribozymes, but the activities of other tRNAVal-driven ribozymes in cultured cells were significantly improved relative to that of the hammerhead ribozyme (Fig. 5A).

It has been suggested that the rate-limiting step in vivo of a reaction mediated by a catalytic RNA, such as a ribozyme, is the substrate-binding step (59, 60). Our analysis is clearly consistent with this suggestion because the efficacies of ribozymes in vivo depend more strongly on the expression system and the localization within cells than on cleavage activities in vitro. It is now apparent that the rate-limiting step in ribozyme-mediated reactions in vivo is not the cleavage step. Thus, hairpin and other ribozymes with limited activities in vitro can have significant inhibitory effects in vivo, as demonstrated previously (9, 11). Even HDV ribozymes, which are very inefficient in vitro, had clear inhibitory effects in cultured cells.

**Cytoplasmic mRNAs Were Cleaved Significantly More Effectively than Nuclear Pre-mRNAs**—Ribozymes expressed under the control of the tRNAVal promoter and the U6 promoter were found, as anticipated, in the cytoplasm and in the nucleus, respectively. The tRNAVal-driven ribozymes that had been exported to the cytoplasm had higher activities than the corresponding tRNAVal-driven ribozymes that remained in the nucleus (Fig. 5). Nuclear pre-mRNAs might be less accessible to ribozymes than cytoplasmic mature mRNAs because pre-mRNAs form complexes with heterogeneous nuclear proteins and small nuclear ribonucleoproteins and interact with various RNA-binding proteins, for example, proteins involved in splicing and in the export of processed mRNAs. It is also likely that higher ordered structures of mRNAs are disrupted more effectively in the cytoplasm by various RNA helicases (60). Thus, a ribozyme can attack its target site during the breathing of the cytoplasmic target mRNA.

The present analysis confirmed that cleavage by various ribozymes occurs more efficiently in the cytoplasm than in the nucleus. Without exception, the tRNAVal-driven ribozymes that had been exported to the cytoplasm (Figs. 3 and 4) had inhibitory effects (Fig. 5A), whereas U6-driven ribozymes that had remained in the nucleus were completely ineffective (Fig. 5B), despite the fact that both types of ribozyme were targeted to the identical site (Fig. 1C) and both had similar activity in vitro (Fig. 2, C and D). By contrast, the EGS in the nucleus mediated cleavage more effectively than the EGS that had been exported to the cytoplasm because the action of the EGS requires RNase P, which is localized in the nucleus. It should be noted also that RNase P might have an RNA unwinding activity.

We confirmed unambiguously that mature mRNAs in the cytoplasm were more accessible to ribozymes than pre-mRNAs in the nucleus. Thus, if we are to exploit ribozyme activity in cells, ribozymes must be concentrated in the cytoplasm while EGSs must remain in the nucleus. Our findings should be useful for the selection of expression systems and the future design of intracellularly active ribozymes.

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