Ribozyme-mediated RNA degradation in nuclei suspension

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ABSTRACT

Ribozymes containing 2'-fluoro- and 2'-amino-modified pyrimidine nucleosides in combination with terminal phosphorothioate linkages were targeted against HTLV-I *tax* RNA. In order to examine the activity of such chemically modified ribozymes in the nuclear environment, they were incubated with nuclei of a Tax-transformed mouse fibroblast cell line. Ribozyme cleavage of *tax* RNA was analyzed by the RNase protection assay. Comparison of the cleavage of *tax* RNA isolated nuclei with that of *tax* RNA present in nuclei suspension revealed a 30 times more efficient cleavage of the latter one. Pre-treatment with proteinase K and SDS abolished the enhancement of the ribozyme-mediated RNA cleavage. Catalytically inactive ribozymes did not yield any cleavage products. These results demonstrate an augmenting effect of nuclear proteins on the ribozyme-mediated RNA cleavage.

INTRODUCTION

The discovery of self-cleaving RNA structures in several plant viroids or satellite RNAs led to the development of sequence-specific, *trans*-cleaving hammerhead and hairpin ribozymes (1–4). Hammerhead ribozymes in particular have gained increasing interest as these ribozymes require a length of only 35–40 nucleotides (nt) and can be easily prepared by automated chemical synthesis (5,6). In addition, site-specific incorporation of several types of chemical modifications have yielded ribozymes with significant stability against nuclease-mediated degradation without seriously affecting their catalytic properties (7–11). Like antisense oligodeoxynucleotides, such modified oligoribonucleotides are suitable for exogenous delivery to cells to interfere with gene expression (12,13). This type of application would create an interesting alternative to gene therapy approaches, where antisense RNA or ribozymes are endogenously

expressed in cells via infected or transfected expression vectors (14–18).

A prerequisite for the application of ribozymes to modulate gene expression is that it reaches the cell compartment where the target RNA is localized and that it cleaves its RNA efficiently. Cellular compartmentalization has been shown to affect the ability of the ribozyme to colocalize with its target RNA for efficient cleavage (19). The catalytic activity of *trans*-cleaving ribozymes is commonly determined in assay systems containing only buffer, divalent cations, a truncated RNA substrate and the ribozyme. Despite valuable information about cleavage kinetics and mechanisms (20,21), these approaches give only limited indications to the activity of a ribozyme in the intracellular milieu. Differences between *in vitro* and *in vivo* secondary structures of the target RNA might dictate different accessibility of the endogenous RNA for ribozyme-mediated cleavage. In addition, the presence of proteins binding to the target RNA or the ribozyme might influence ribozyme catalysis. So far a limited number of reports have been published where the cleavage ability of ribozymes was examined in the presence of single proteins (22–24). These studies demonstrated an enhancement of the ribozyme cleavage reaction by RNA-binding proteins like hnRNP A1 or the capsid protein NC7 of HIV-1. However, higher than optimal concentrations of both an hnRNP A1 derived peptide and NC7 inhibit the RNA cleavage (24). Therefore, the question remains if an intracellular ribonucleoprotein–RNA complex is more accessible to ribozyme-mediated cleavage than free RNA. This question prompted us to investigate the cleavage activity of ribozymes in a system which contains the naturally occurring RNA–protein complexes.

In the present study the ribozyme-mediated cleavage of HTLV-1 *tax* RNA in nuclei suspension was investigated. It is shown that ribozymes cleave their target RNA in this cellular compartment and that, at least for the particular ribozyme and target combination tested, the ribozyme efficiency is enhanced by more than one order of magnitude in the nuclear environment compared to the cleavage of isolated RNA.

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MATERIALS AND METHODS

Materials

T7 RNA polymerase was obtained from US Biochemicals. Nucleases were supplied by Boehringer Mannheim, all other enzymes were purchased from Promega. Radioactively labeled nucleoside triphosphates were supplied from NEN-Du Pont. All other chemicals were of analytical grade.

Plasmids and oligonucleotides

Plasmids pGEM3 and pGEM4 were purchased from Promega. The run-off transcription vectors pGEM3tax and pGEM4tax were obtained by cloning the 186 bp long *HindIII/ClaI* fragment from pHTLVtat1 (25) into either pGEM3 or pGEM4 using the *HindIII* and *AccI* site of the multiple cloning site. Oligonucleotides were synthesized and purified as described (11). The oligonucleotide sequences were 5'-A*FC^FC FC^FUG G^FCNU GANU GAG G^FC^FC GFU^FU AGG FC^FCG AAA AG^FU *G*G*G^{3'} [TAXRE(F,N,S)], 5'-A*FC^FC FC^FUG G^FCNU AANU GAG G^FC^FC GFU^FU AGG FC^FCG AAA AG^FU *G*G*G^{3'} [TAXIR(F,N,S)] and 5'-G*GG FUGA A^FCNU GANU GAG G^FC^FC GFU^FU AGG FC^FCG AAG G^FUFC *FC*FC*A^{3'} [TAXSC(F,N,S)]. 2'-Amino-2'-deoxyuridines are indicated by 'N', 2'-fluoro-2'-deoxynucleotides by 'F' and phosphorothioate linkages by '*'.

Cell culture

The fibroblastic B cell line derived from LTRtax transgenic C57BL/6 mice (26) was grown in DMEM supplemented with 10% fetal calf serum, 4 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO₂.

Preparation of total cell RNA and cell nuclei

Total cellular RNA from B cells was isolated by the single-step guanidinium method (27). For the isolation of B cell nuclei, two confluent T150 flasks were trypsinized, washed with saline, and resuspended in 500 µl hypotonic buffer [10 mM Tris-Cl pH 7.5, 10 mM NaCl, 5 mM MgCl₂, 0.3% NP-40, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. After 40 strokes with a dounce B homogenizer the nuclei were pelleted by centrifugation at 500 g at 4°C for 5 min. After washing with 500 µl hypotonic buffer the pellet was resuspended in 400 µl storage buffer (50 mM Tris-Cl pH 7.5, 5 mM MgCl₂, 40% glycerol) and the protein concentration was determined by the Bradford assay (28). Typical concentrations were between 15 and 25 µg/µl. The nuclei were aliquoted, frozen on dry ice and stored at -70°C.

Run-off transcription

The plasmids pGEM3tax and pGEM4tax were linearized with *HindIII* or *BamHI*, respectively. Run-off-transcriptions were performed in the presence of 50 ng/µl linearized plasmid, 500 µM of each NTP, 40 mM Tris-Cl pH 7.5, 6 mM MgCl₂, 5 mM DTT, 1 mM spermidine, 8 µCi/µl [α -³²P]UTP and 0.5 U/µl T7 RNA polymerase in a total volume of 20 µl. After incubation at 37°C for 30 min, RNase-free DNase I was added to a final concentration of 1 U/µl and the solution was further incubated for 15 min followed by phenol-chloroform extraction and passage through

a Sephadex-G50 spin column. The RNA was ethanol precipitated and the pellet was redissolved in water. The 228 nt run-off transcript obtained from pGEM3tax contains the *tax* antisense sequence from position 454 to 269 of the mRNA, the 209 nt long transcript from pGEM4tax the corresponding sense RNA. For the synthesis of RNA probes for RNase protection assays, the unlabeled UTP was omitted. After DNase I treatment, the RNA was ethanol precipitated and loaded on a 6% acrylamide-8 M urea gel. The full length product was excised, eluted by shaking for 2 h at 65°C in 2 M sodium acetate pH 6.0 and ethanol precipitated. The pellet was redissolved in hybridization buffer {40 mM piperazine-*N,N'*-bis[2-ethanesulfonic acid] (PIPES) pH 6.4, 400 mM NaCl, 1 mM EDTA} and stored at -20°C.

Ribozyme reactions

The cleavage kinetics with *tax*RNA obtained by run-off transcription were performed and analysed as described with a Pharmacia LKB Ultrascan XL Laserscanner (8,11). Cleavage reactions with isolated RNA contained 25 mM Tris-Cl pH 7.5, 100 mM KCl, 10 mM MgCl₂, 300 ng/µl RNA and 0.1–2.5 µM ribozyme. After incubation for 6–7 h at 37°C, the reaction was stopped by addition of 2 µl 500 mM EDTA; the RNA was ethanol precipitated and analyzed by RNase protection assay. Incubation of ribozymes with cell nuclei was performed in the presence of 25 mM Tris-Cl pH 7.5, 100 mM KCl, 10 mM MgCl₂, nuclei (8–12.5 µg protein/µl) and 50 nM to 1 µM ribozyme. After incubation for ≤1 h at 37°C, the reaction was stopped by addition of 2 µl 500 mM EDTA, the RNA was isolated as described (27), and analyzed by the RNase protection assay.

To examine directly the influence of proteins on the ribozyme reaction, nuclei suspensions (final concentration 9.3 mg/ml) were incubated in 14.3 mM MgCl₂ and 143 mM KCl for 15 min at room temperature followed by centrifugation for 3 min at 500 g. This allowed the RNA-protein complex to diffuse out of the nuclei. To degrade proteins, supernatant was incubated with Proteinase K and SDS [final concentrations of 100 µg/ml and 0.5% (w/v), respectively] for 15 min at 37°C. Cleavage reactions were performed as described above by addition of the ribozyme TAXRE(F,N,S) to a final concentration of 1 µM and analyzed by RNase protection.

To determine the location of the cleavage reaction, 1 µM TAXRE(F,N,S) was incubated with nuclei suspension as described above. Aliquots of 20 µl were withdrawn at the indicated time points and added to 2 µl 500 mM EDTA followed by centrifugation at 2000 g for 3 min. The pellets containing the nuclei and the supernatants were separately analyzed by the RNase protection assay.

Apparent kinetic constants under single turnover conditions were obtained by plotting the observed cleavage rate k_{obs} against the quotient of k_{obs} over the total ribozyme concentration $[E]_t$ according to equation 1 (11):

$$-\frac{\ln \frac{[S]_t}{[S]_0}}{t} = k_{\text{obs}} = -K_m \frac{k_{\text{obs}}}{[E]_t} + k_{\text{react}} \quad (1)$$

The reaction rate k_{obs} equals the negative natural logarithm of the remaining fraction of uncleaved RNA ($[S]_t/[S]_0$) divided by the reaction time t . The negative slope of the plot represents the K_m

Figure 1. Structure of TAXRE(F,N,S). The ribozyme is shown complexed to the *tax* RNA. The start of translation (position 280 of the *tax* mRNA) is underlined and the cleavage site is indicated by an arrow. Cleavage takes place 3' of position 23 of *tax* run-off transcript and of position 291 of the *tax*-encoding mRNA. 2'-Fluoropyrimidine nucleosides are circled, whereas the 2'-aminouridines at position 4 and 7 are boxed (numbering according to ref. 39). Phosphorothioate linkages are indicated by an 'S'.

value, and the intercept of the regression line with the ordinate gives the maximal reaction rate k_{react} .

Curve fitting of time courses for ribozyme-mediated cleavages was performed according to either the exponential equation 2

$$\frac{[S]_t}{[S]_0} = e^{-k_t} \quad (2)$$

or the biexponential equation 3

$$\frac{[S_1]_t + [S_2]_t}{[S_1]_0 + [S_2]_0} = \frac{[S_1]_0}{[S_1]_0 + [S_2]_0} e^{-k_1 t} + \frac{[S_2]_0}{[S_1]_0 + [S_2]_0} e^{-k_2 t} \quad (3)$$

where k_1 and k_2 represent the cleavage rates for the RNA species S_1 and S_2 , respectively.

RNase protection assay

RNase protection assays were performed as described (27) except that the hybridization volume was reduced to 15 μ l, the hybridization temperature was 60°C and the RNase digestion was performed in a total volume of 175 μ l. The 227 nt long run-off transcript obtained with pGEM3tax served as the antisense probe. Protected fragments were separated on a 10% polyacrylamide gel containing 8 M urea, analyzed by autoradiography and quantitated by laser scanning densitometry as described above.

RESULTS

Design of chemically modified ribozymes targeted against *tax* RNA

In previous studies, antisense inhibition of the HTLV-1 *tax* protein expression in a transgenic mouse model has been demonstrated. In these studies, an AUG bracketing oligodeoxynucleotide was shown to be highly effective (26). In the present studies we have generated a hammerhead ribozyme directed against a site 9 nt downstream of the AUG start codon (Fig. 1). As this site is downstream of the synthetic linker site

Figure 2. Degradation of nuclear and isolated *tax* RNA by chemically modified ribozymes. Ribozymes were incubated either with cell nuclei or with purified total RNA isolated from nuclei of *tax*-transformed fibroblasts as described in Materials and Methods. The reaction time for the cleavage of the nuclear RNA was 1 h, for the cleavage of the isolated RNA 7 h. The concentration of each oligonucleotide was 1 μ M. The nuclear RNA was isolated and analyzed by RNase protection. The ribozyme cleavage product ('P') is 22 nt shorter than the uncleaved RNA ('S').

present in the original transgene construct (25), this sequence is fully contained within the human viral transcript.

Each of the ribozymes contains four terminal phosphorothioate internucleotidic linkages (one at the 5'-terminus and three at the 3'-terminus) in combination with 2'-fluoropyrimidine nucleosides except for positions 4 and 7 in the conserved core region which are 2'-aminouridines. This combination of chemical modifications has been shown to confer high stability in serum without decreasing the catalytic efficiency of the ribozyme (11). The active ribozyme with the combination of fluoro, amino and phosphorothioate modifications is termed TAXRE(F,N,S) (Fig. 1). In one ribozyme control, G⁵ of the conserved hammerhead core region was replaced by an A, which abolishes catalytic activity (29). This oligoribonucleotide, TAXIR(F,N,S), served as an antisense control because it still can bind to the ribozyme target sequence. For the second control oligoribonucleotide, the orientation of the sequences complementary to the target region were reversed [TAXSC(F,N,S)]. Therefore, this ribozyme is incapable of binding in a sequence-specific manner to the *tax*RNA and served as a sequence specificity control in the ribozyme assays.

The cleavage activity of the ribozyme was examined initially with a 209 nt long run-off transcript from pGEM4tax containing the N-terminal coding region of the *tax* mRNA. Incubation of this RNA with the active ribozyme TAXRE(F,N,S) yielded the expected cleavage products of 183 and 26 nt in length while the inactive control ribozyme TAXIR(F,N,S) showed no activity (data not shown). Evaluation of the time and concentration dependence of the cleavage reaction under single-turnover conditions revealed a k_{react} of $0.54 \times 10^{-3} \text{ s}^{-1}$, a K_m of 980 nM and a catalytic efficiency k_{react}/K_m of $550 \text{ M}^{-1} \text{ s}^{-1}$.

The nuclear environment enhances ribozyme catalysis

We compared ribozyme-mediated cleavage of *tax* RNA as part of isolated total nuclear RNA to that present in cell nuclei (Fig. 2). Ribozymes were incubated either with RNA isolated from nuclei for 7 h or with cell nuclei for 1 h at 37°C followed by RNA

Figure 3. Influence of nuclear proteins on ribozyme-mediated RNA cleavage. Internally ^{32}P -labeled *tax* run-off transcripts or nuclear supernatant were pretreated with proteinase K and SDS prior to addition of TAXRE(F,N,S) at a final concentration of $1\ \mu\text{M}$. (a) Influence of KCl, proteinase K and SDS on the ribozyme-mediated cleavage of ^{32}P -labeled *tax* run-off transcript. Open circles, cleavage in the presence of Tris-Cl and MgCl_2 ; closed circles, as before but with additional 100 mM KCl; open diamonds, as before but treated with proteinase K and SDS. (b) Influence of proteinase K and SDS on the cleavage reaction in nuclear supernatant. RNA cleavage was analyzed by RNase protection. Open circles, untreated supernatant; closed circles, supernatant treated with proteinase K and SDS.

isolation. RNA cleavage analysis was performed with an RNase protection assay using a 228 nt long *tax* antisense transcript as probe. This probe is complementary to the *tax* sense RNA region ranging from 22 nt upstream to 164 nt downstream of the ribozyme cleavage site. Cleavage was observed only for the catalytically active ribozyme TAXRE(F,N,S) (Fig. 2). Comparison of TAXRE(F,N,S)-mediated cleavage of isolated RNA with that of RNA in cell nuclei suspension revealed the same pattern. However, TAXRE(F,N,S) cleaved the *tax* RNA in the nuclei suspension more efficiently than the *tax* RNA present in the isolated RNA. Neither the catalytically inactive TAXIR(F,N,S) nor the sequence specificity control TAXSC(F,N,S) yielded any cleavage fragments. These findings demonstrate that the appearance of the product band is directly correlated with the cleavage activity by the ribozyme and is therefore neither an artefact of the RNase protection assay nor due to RNA degradation directly induced by nuclear proteins.

Table 1. Comparison of ribozyme activities with isolated RNA and in cell nuclei

Cleavage of	k_{react} ($10^{-3}\ \text{s}^{-1}$)	K_m (nM)	k_{react}/K_m ($\text{M}^{-1}\ \text{s}^{-1}$)
isolated RNA ^a	0.023	670	34
nuclear RNA ^b	0.14	150	950

^aRNA was isolated from cell nuclei prior to the cleavage reaction. Ribozyme concentrations ranged from 100 nM to $2\ \mu\text{M}$; reactions were stopped after 6 h and analyzed by RNase protection.

^bRibozyme concentrations ranged from 50 nM to $1\ \mu\text{M}$; reactions were stopped after 1 h and analyzed by RNase protection.

The dependence of cleavage on ribozyme concentration was analyzed to obtain apparent kinetic constants for ribozyme-mediated *tax* RNA cleavage (Table 1). All cleavage reactions were analyzed by RNase protection. Ribozyme-mediated cleavage of RNA isolated from nuclei before addition of the ribozyme proceeded with an apparent k_{react} of $2.3 \times 10^{-5}\ \text{s}^{-1}$ and an apparent

K_m of 670 nM. The *tax* RNA present in the nuclei suspension was cleaved with an apparent k_{react} of $1.4 \times 10^{-4}\ \text{s}^{-1}$ and an apparent K_m of 150 nM. Thus, as indicated by the resulting k_{react}/K_m values, TAXRE(F,N,S) cleaved *tax* RNA present in nuclei suspension 30 times more efficiently than *tax* RNA present in isolated RNA.

Pretreatment of nuclei suspension with Proteinase K and SDS abolishes enhancement of ribozyme-mediated cleavage

In order to demonstrate more directly protein enhancement of the ribozyme reaction, cleavage of RNA-protein complexes present in the supernatant of nuclei was compared with that of supernatant pre-treated with proteinase K and SDS. Pretreatment of the *tax* run-off transcript did not affect its ribozyme-mediated cleavage, demonstrating that neither SDS nor proteinase K in the concentrations used here interfere with ribozyme catalysis in the absence of nuclear proteins (Fig. 3). However, when nuclei supernatant was pretreated with proteinase K and SDS, ribozyme-mediated RNA cleavage proceeded much slower than the cleavage in untreated nuclei supernatant (Fig. 3). Curve fitting according to equation 2 revealed an apparent cleavage rate of $4.3 \times 10^{-5}\ \text{s}^{-1}$, which is very similar to the maximal cleavage rate k_{react} of $2.3 \times 10^{-5}\ \text{s}^{-1}$ obtained for isolated RNA (Table 1). In contrast, cleavage of target RNA present in untreated supernatant proceeded according to the biexponential equation 3 suggesting the presence of at least two different species of *tax* RNA (Fig. 2). Some 40% of the total *tax* RNA belongs to a 'fast' species S_1 which was cleaved by TAXRE(F,N,S) with a rate k_1 of $2.2 \times 10^{-3}\ \text{s}^{-1}$. Thus, this species was cleaved 50-fold faster than the *tax* RNA of the supernatant treated with proteinase K and SDS. The 'slow' species S_2 , which is some 60% of the total *tax* RNA, was cleaved with a rate k_2 of $7.2 \times 10^{-5}\ \text{s}^{-1}$, which is close to the rate obtained for the pretreated supernatant. The similar cleavage rates obtained for the 'slow' species and the target RNA present in the pretreated supernatant suggests that the former one is already

partially stripped of proteins and might, therefore, not represent the native mRNA-protein particle.

TAXRE(F,N,S) cleaves *tax* RNA inside and outside the nuclei

An important question when working with nuclei suspension is the location of the cleavage reaction. Either the ribozyme cleaves its target inside the nucleus or, alternatively, it remains outside and cleaves only that fraction of the target RNA which leaves the nuclei. In order to clarify this point, aliquots were withdrawn from the cleavage reaction, separated into nuclei and supernatant by centrifugation, and analyzed separately for cleavage products. Immediately after assembly of the reaction mixture a significant amount of *tax* RNA was present in the supernatant (Fig. 4). During the incubation time, increasing amounts of cleavage product accumulated in both fractions. Therefore, the ribozyme is capable of entering the nucleus and of cleaving its target RNA intranuclearly. Consistent with the results described above, cleavage is also observed in the supernatant and is promoted to a similar extent as in the nuclei fraction. These results are supported by the finding, that after 1 h of incubation some 20% of radioactively labeled ribozyme was associated with the nuclei, whereas the main ribozyme fraction remained in the supernatant (data not shown).

DISCUSSION

Hammerhead ribozymes have gained increasing attention for therapeutic applications to modulate sequence-specific gene expression (14–18,30). Studies of intracellular localization of oligodeoxynucleotides suggest that exogenously applied oligonucleotides including ribozymes are enriched in cytosolic organelles and in the nucleus (31–34). This has also been demonstrated for ribozymes (35,38). Therefore, we were interested in the effect of the cell nuclear environment on ribozyme activity. As a target region we chose a sequence just downstream of the start of translation for the HTLV-1 *tax* RNA (Fig. 1). This site was already successfully targeted by an antisense oligodeoxynucleotide (26) and was therefore expected to be accessible for ribozyme-mediated cleavage. Cleavage kinetics using a 209 nt long run-off transcript containing the target sequence demonstrated the cleavability of this site by a ribozyme.

Comparison of ribozyme-mediated cleavage of short RNA substrates with that of longer RNAs reveals several orders of magnitude lower cleavage efficiency for the long substrate. This difference has been attributed to the lower accessibility of the longer, more structured RNAs for the binding of the ribozyme (8,11,36,37). When TAXRE(F,N,S) was incubated with isolated total nuclear RNA, cleavage of the endogenous target RNA was 15 times less efficient than that of a 209 nt long run-off transcript. The lower efficiency might be due to either a different secondary or tertiary structure of the endogenous *tax* RNA or to competition between the target and partially homologous RNA sequences for the ribozyme binding.

However, proteins present in the nuclei suspension facilitate the ribozyme-mediated cleavage of the endogenous *tax* RNA resulting in a 30-fold higher catalytic efficiency compared with the cleavage of the isolated RNA (Table 1). Pretreatment of nuclear supernatant with proteinase K and SDS abolishes this enhancement (Fig. 3). The similarity of the cleavage rates for pretreated

Figure 4. Ribozyme-mediated RNA cleavage inside and outside of cell nuclei. The ribozyme TAXRE(F,N,S) (1 μ M) was incubated with nuclei suspension from Tax-transformed mouse fibroblasts for 1 h. Aliquots were withdrawn at the indicated time points and fractionated by centrifugation into nuclei pellet and supernatant. Both fractions were separately analyzed for full-length and cleaved *tax* RNA by the RNase protection assay. (a) Autoradiograph of the cleavage reaction. (b) Graphical representation of the time course. Open circles, cleavage in the nuclei pellet; filled circles, cleavage in the supernatant.

supernatant and isolated RNA demonstrates, that the low cleavage of the latter one is not due to inhibition by guanidinium ions.

RNA cleavage by the ribozyme is enhanced both within the cell nuclei and in the supernatant of nuclei (Fig. 4). These findings exclude the possibility that the observed enhancement in nuclei suspension is due to a locally increased ribozyme concentration inside the nuclei. Instead they suggest that not only the RNA but also the proteins responsible for the enhancement leave the nuclei either independent of or complexed to the *tax* RNA. The increased efficiency indicates a greater accessibility of the *tax* RNA to ribozyme-mediated cleavage in the presence of nuclear proteins. It has recently been shown that the HIV p7 nucleocapsid protein as well as the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) are able to facilitate ribozyme reactions at optimal concentrations (22–24). In particular both proteins facilitate the formation of the ribozyme-substrate complex, probably by unfolding the target RNA. Thus, nuclear proteins might facilitate the ribozyme-mediated cleavage by the same mechanism. In addition, proteins bound to RNA sequences partially homologous to the target sequence might prevent the binding of ribozyme to such sequences and thereby prevent the

formation of uncleavable ribozyme–RNA complexes. Thus, the increased ribozyme-mediated cleavage can be explained by the unfolding of the target sequence and the promotion of correct ribozyme binding by RNA binding proteins.

In summary, we show that ribozymes are able to find and cleave their target RNA in isolated cell nuclei. The cleavage reaction is greatly increased by nuclear proteins, though strictly dependent on the catalytic activity of the ribozyme. The results suggest the idea that intracellular target RNAs are more susceptible to ribozyme-mediated cleavage than implied by *in vitro* experiments using isolated cellular RNA as substrate. In addition, this simple system utilizing isolated cell nuclei should be very useful for optimizing many other oligonucleotide-based approaches to inhibit gene expression such as antisense, triplex and decoy strategies, in addition to ribozymes.

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