Mechanistic Studies of Hammerhead Ribozymes and their Applications *in vivo*

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1. Hammerhead ribozymes

The word "ribozyme" is derived from the words **ribo**nucleic acid (RNA) and en**zyme**, and it denotes an RNA molecule with catalytic properties. Catalytic RNAs include Group I and II introns; the RNA subunit of RNase P; hammerhead, hairpin and hepatitis delta virus ribozymes; and ribosomal RNA (1-5). Among these catalytic RNAs, the hammerhead ribozyme is the smallest. Naturally occurring hammerhead ribozymes were found within RNA viruses and they act "in *cis*" during viral replication by the rolling circle mechanism (4-6). From the profile of the secondary structure of the catalytic domain, this type of ribozyme is called a "hammerhead" ribozyme (Fig. 1a). Hammerhead ribozymes have been engineered in such a way they can act "in *trans*" against other RNA molecules (7-8). The *trans*-acting hammerhead ribozyme developed by Haseloff and Gerlach (8) consists of an antisense section (stems I and III) and a catalytic domain with a flanking stem II and loop section (Fig. 1b). Since the first demonstration by Sarver and Rossi's group of successful inhibition of the replication of HIV-1 by ribozymes in cultured cells (hereafter, the term ribozymes refers exclusively to hammerhead-type ribozymes unless otherwise noted), ribozymes have been extensively investigated not only in terms of the mechanism of their actions but also their possible application *in vivo* (9-11).

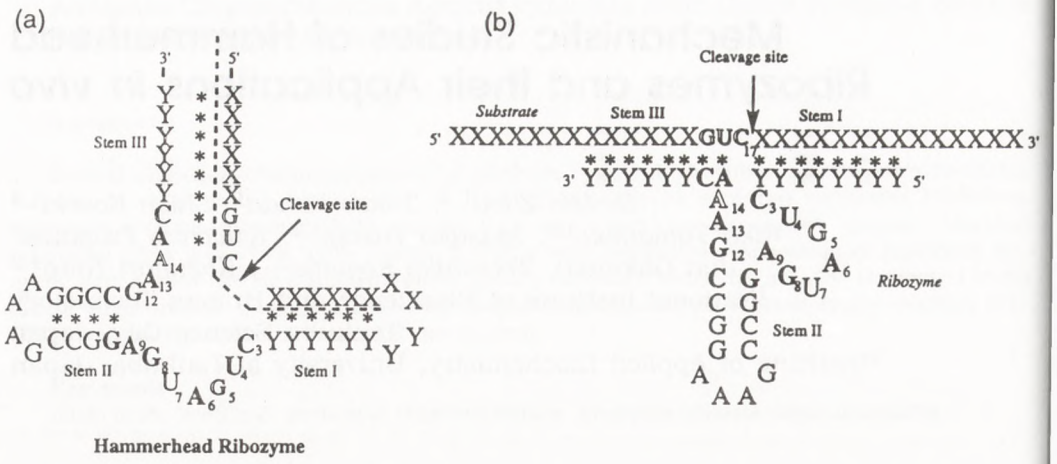

Fig. 1. Naturally occurring *cis*-acting (a) and engineered *trans*-acting (b) hammerhead ribozymes.

2. Cleavage sites (NUX triplets) of hammerhead ribozymes

In order to define the sequence requirements for an active structure, extensive mutagenesis studies of the conserved region have been performed in both *cis* and *trans* reaction systems (12-17). Figure 1b shows a *trans* system in which the binding sites indicated by Ys are complementary to regions of Xs in the substrate RNA (stem I and stem III). The arrow after the GUC triplet shows the site of cleavage. Several mutagenesis studies were carried out to examine the importance of the conserved trinucleotide GUC at the cleavage site 12-17. Early results revealed that G at the third position in the triplet, which might extend stem I by forming a G_{17}:C_3 pair [the numbering of the bases is that of Hertel et al. (18)], inhibited the cleavage reaction in all but one case and that U at the central position was required for efficient cleavage (12,14,15). These observations led to the generally accepted NUX rule (where N can be A, U, G or C; and X can be A, U or C) which states that a substrate with a NUX triplet can be cleaved by a hammerhead ribozyme. However, there were some inconsistencies among the results reported by different groups. For example, a substrate that contained an AUC triplet was cleaved in one study with an efficiency similar to cleavage of the wild-type substrate (14) while, in other studies, the same substrate was cleaved with much lower efficiency (12) or not at all (15). The differences could have been due not only to differences among reaction systems, which include the type of reaction (one *cis* and two different types of *trans* reactions), the sequence of the hammerhead complex, and the reaction conditions, but also to the experimental design: in every case only a simple comparison of rates of cleavage of certain substrates at fixed concentrations was

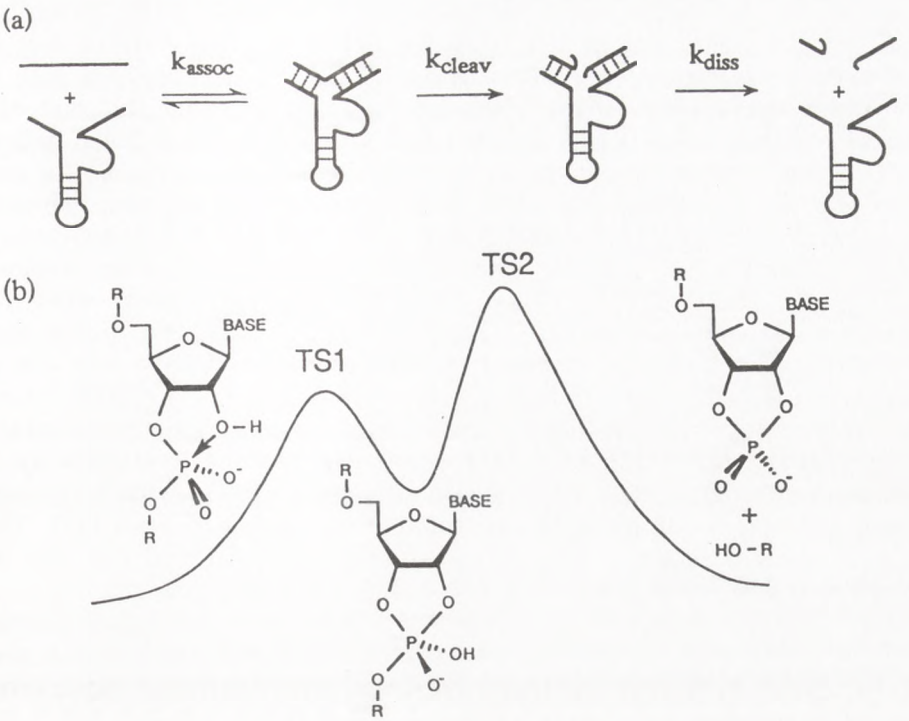

Fig. 2. (a) Minimal reaction scheme for hammerhead ribozymes. The reaction catalyzed by the hammerhead ribozyme consists of at least three steps. The substrate (together with Mg^{2+} ions) first binds to the ribozyme (k_{assoc}). The phosphodiester bond of the bound substrate is cleaved by the action of Mg^{2+} ions (k_{cleav}). The cleaved fragments dissociate from the ribozyme and the liberated ribozyme is now available for a new series of catalytic events (k_{diss}). (b) Schematic diagram of the RNA-cleaving reaction. According to molecular orbital calculations, **TS2** is a higher-energy state than **TS1**.

performed. Even though earlier results were obtained with ribozymes at a 1.3- to 1.5-fold molar excess, relative to the substrate, the observed rate of cleavage would have been lower than the maximum rate (k_{cat}) unless the total concentration of substrate and ribozyme were high enough with respect to the K_m value. In other words, the determined rate constants could reflect values of either k_{cat} or k_{cat}/K_m, depending on the concentrations of substrate and ribozyme used in the experiments. Therefore, it became important to determine whether a reduction in rate was caused by a reduced value of k_{cat} or by an increase in K_m. Determination of individual values of k_{cat} and K_m appeared to be essential for an objective discussion of differences in susceptibility among various mutant forms of the cleavage site (16).

From a practical point of view, since *trans* reactions are suitable for targeting specific RNAs (8,9), a detailed examination of the NUX rule in *trans* systems should help us to choose appropriate target sequences. Therefore, in order to investigate the range of cleavage activities at NUX triplets,

we performed detailed kinetic analysis of all possible NUX mutants by measuring individual values of k_{cat} and K_m (16). We chose an 11-mer substrate with the sequence of 5'-GCCNUXCCCCG-3'. The observed rate constant under substrate-saturating conditions (k_{cat}) reflected the rate of the chemical cleavage step (k_{cleav} in Fig. 2a). Our detailed analysis indicated that GUC was cleaved most efficiently under conditions suitable for measurements of k_{cat} and k_{cat}/K_m, with CUC and UUC coming next. Therefore, when a target site in a *trans*-acting system is chosen, GUC or CUC may be preferred. However, in *cis*-acting systems, in which K_m values are irrelevant, other triplets, such as AUC, GUA, and AUA, may be chosen since these triplets are associated with high values of k_{cat}. In fact, the minus strand of the virusoid of Lucerne transient streak virus, (-) vLTSV, and the plus strand of the satellite RNA of barley yellow dwarf virus, (+) sBYDV, use the GUA triplet and the AUA triplet, respectively, for hammerhead-catalyzed cleavage during their replication. Independent mutagenesis studies by Zoumadakis and Tabler of NUX triplets in a *cis*-acting system with a completely different binding sequence yielded similar conclusions to ours (17). Therefore, it is likely that the conclusion is universally valid and not just a sequence-dependent result (16).

3. A reaction scheme for trans-acting hammerhead ribozymes

The *trans*-acting hammerhead ribozyme consists of an antisense section (stems I and III) and a catalytic domain with a flanking stem II and loop section (Fig. 1b). The minimum reaction scheme can be described as shown schematically in Fig. 2a. First, the substrate (together with Mg^{2+} ions) binds to the ribozyme to form a Michaelis-Menten complex via formation of base pairs with stems I and III (k_{assoc}). Then, a specific phosphodiester bond in the bound substrate is cleaved by the action of Mg^{2+} ions [k_{cleav}; the ribozyme functions as a metalloenzyme (5,19,26)]. This cleavage generates products with 2',3'-cyclic phosphate and 5'-hydroxyl groups (Fig. 2b). Finally, the cleaved fragments dissociate from the ribozyme and the liberated ribozyme is now available for a new series of catalytic events (k_{diss}). Individual rate constants for a complete kinetic scheme were reported by Uhlenbeck's group (27). As can be seen in Fig. 2b, the nucleophile in the reactions catalyzed by the hammerhead, hairpin and HDV ribozymes is a 2'-alkoxide, resembling the nucleophile in reactions catalyzed by proteinaseous ribonucleases. Therefore, hammerhead ribozymes are incapable of cleaving DNA substrates.

A change in the rate-determining step was detected from an Arrhenius plot after measurements of the rate constant of the reaction (k), with the same 11-mer substrate (called R11) as that used for the mutagenesis study (5'-GCCGUCCCCCG-3'), at different temperatures and plotting log k *versus* 1/T (28). At 25-50°C, the chemical cleavage step (k_{cleav}) was clearly the rate-determining step because no burst kinetics were detected at the meas-

urement temperature of 37°C (29). Therefore, at mid-range temperatures, the cleaved fragments dissociated from the ribozyme at a higher rate than the rate of the chemical reaction ($k_{cleav} < k_{diss}$). When the temperature was below 25°C, the cleaved fragments adhered to the ribozyme more tightly and the product-dissociation step became the rate-determining step. Above 50°C, the rate of the reaction decreased, perhaps because, at such high temperatures, the formation of the Michaelis-Menten complex was hampered by thermal melting. The melting temperature (T_m) of stem II of this ribozyme was above 80°C.

Transition temperatures depend heavily on the length of the binding arms (stems I and III). When longer binding arms are used, the product-dissociation step (k_{diss}) becomes the rate-determining step even at temperatures close to 37°C. Therefore, it is not always advantageous to choose long binding arms, especially *in vitro*. However, *in vivo*, there may exist proteins that facilitate the unwinding of the RNA-duplex and, thus, use of longer binding arms might be feasible (30-32).

4. Mechanism of action of hammerhead ribozymes as metalloenzymes

The chemical cleavage step shown in Fig. 2a (k_{cleav}) probably consists of two steps, namely, attack by the 2'-oxygen and the departure of the 5'-oxygen via two separate transition states, **TS1** and **TS2** (Fig. 2b). Alternatively, as in enzyme-catalyzed reactions, **TS1** and **TS2** could be sufficiently stabilized by acid/base catalysis that leads to a concerted reaction with a single transition state. In the case of RNase A, the reaction is initiated by His12, which acts as a base catalyst by abstracting a proton from 2'-OH. Then, the resulting, more nucleophilic 2'-oxyanion attacks phosphorus to generate a pentacoordinate intermediate/transition state. Finally, His119 acts as an acid catalyst by supplying a proton to the leaving 5'-oxygen, with resultant efficient cleavage of the exocyclic P-O(5') bond. Of the two transition states (**TS1** and **TS2**), our molecular orbital calculations indicate that **TS2** is always a higher-energy state than **TS1** (25, 33-40). Thus, for catalysis of cleavage of RNA, it is mandatory to stabilize **TS2**, with the 5'-leaving oxygen (33). Uhlenbeck's group demonstrated that one "Mg^{2+} ion-moiety" (see below) catalyzes the formation of **TS1**, namely, the abstraction of a proton from 2'-OH (19). Another Mg^{2+} ion-moiety could catalyze the second step (**TS2**) as well (5,26,41,42).

What is the Mg^{2+} ion-moiety? Does it correspond to water-hydrated Mg^{2+} ions (5) or to Mg^{2+} ions that become directly coordinate of to the attacking and leaving oxygen moieties (23,26,33,41,42)? In order to distinguish between these two possibilities, we examined solvent isotope effects. If water-hydrated Mg^{2+} ions were to display the amphoteric properties necessary for supply or abstraction of a proton, the movement of a proton in the transition

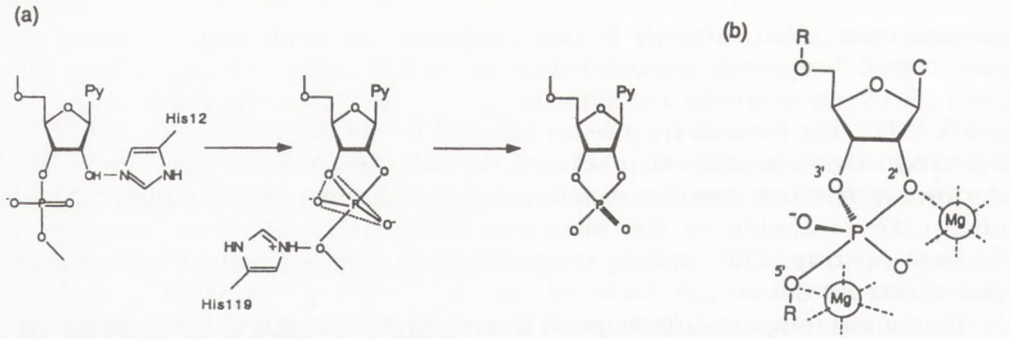

Fig. 3. (a) Reaction mechanism for the cleavage of a phosphodiester bond by RNase A. (b) Catalytic magnesium ions at the cleavage site of a hammerhead ribozyme catalyze the reaction by directly coordinating to attacking and leaving oxygens.

state should be associated with significant solvent isotope effects (43). We failed to find any isotope effect, after correction for the difference in pK_a for a water molecule coordinated to a Mg^{2+} ion, in a reaction with the above-mentioned R11 substrate. The observed rate constant (k_{cat}) reflected the rate of the cleavage process (k_{cleav}). Since reactions catalyzed by hammerhead ribozymes take place at the same rate in H_2O and D_2O, we need to propose a mechanism in which no proton transfer occurs at the transition state (26, 41,42). A proposed mechanism is shown in Fig. 3b, in which Mg^{2+} ions are directly coordinated to the attacking and leaving oxygens and no proton transfer is involved. This mechanism can rationalizes the absence of a kinetic isotope effect not only for the stepwise mechanism but also for the concerted process (25,26). More recently, we synthesized, for the first time, a natural all-RNA substrate that contains a 5'-thio-leaving group at the cleavage site and performed detailed kinetic analysis (42). In contrast to the earlier conclusion based on similar reactions with the DNA substrate (44), our kinetic analysis revealed that hammerhead ribozymes exploit the general double-metal-ion mechanism of catalysis, wherein metal ions are coordinated directly to the attacking and leaving oxygens. This is the first demonstration of double-metal-ion catalysis in hammerhead reactions.

In general, RNAs have few functional groups that can potentially act as acid/base catalysts, as compared with proteins (Fig. 3a). By adapting metal ions as the true catalysts at the active site (Fig. 3b), RNA enzymes can overcome this disadvantage. It is, thus, possible that ribozymes in general exploit the general double-metal-ion mechanism of catalysis (23).

We must add here, however, that our analyses by NMR of the hammerhead ribozyme indicated that Mg^{2+} ions are not only the true catalysts but also play a role in establishing the active form of the ribozyme-substrate complex (45). It remains to be determined whether the catalytically indispensable Mg^{2+} ions are the same as the structurally important Mg^{2+} ions.

5. Detection of undegraded oligonucleotides *in vivo* by fluorescence resonance energy transfer

In the search for a treatment for AIDS and other diseases, ribozymes or their artificially modified analogues appear to be good candidates for drugs of the future since specially designed ribozymes can specifically cleave the mRNA of the AIDS virus (9,10). In studies of such possible therapeutic modalities, the structural integrity of oligonucleotides merits careful consideration because of the dependence of specific binding activity on the length of the oligonucleotide sequence. There is always the undesirable possibility of digestion of oligonucleotides by intracellular nucleases. Ribozymes are especially sensitive to intracellular ribonucleases because their active sites are composed of RNA (46). If we are to monitor the fate of ribozyme *in vivo* by use of conventional materials labeled with a fluorescent dye at one end, we are likely to detect degraded RNAs labeled with a fluorescent dye at their end. To address these issues, we investigated methods for estimating the integrity of injected oligonucleotides and detecting the nucleolytic activity in living cells using oligonucleotides that had been double-labeled with two fluorescent dyes. We examined the fluorescence characteristics of and the fluorescence resonance energy transfer (FRET) within these double-labeled oligonucleotides (Fig. 4) (47).

FRET is an interesting example of a fluorescence-related phenomenon. When the fluorescence spectrum of one fluorochromes, the donor, overlaps with the excitation spectrum of another fluorochrome, the acceptor, and when the donor and the acceptor are in close physical proximity, the excitation of the donor induces the emission of fluorescence from the acceptor as if the acceptor has been excited directly and the intensity of fluorescence from the donor decreases. The extent of FRET is extremely sensitive to the distance between the donor and the acceptor, being inversely proportional to the sixth power of the distance. This phenomenon can be explored for studies of intermolecular and intramolecular relationships in biophysical investigations and in cell biology.

To examine the possibility of visualization of FRET in cells, double-labeled oligonucleotides with fluorescein and rhodamine X (F-ODN-R) were microinjected into unfertilized sea urchin eggs. These eggs were illuminated with blue light at 470-490 nm to photo-excite the fluorescein fluorochrome of the F-ODN-R. The green and red components of the emitted fluorescence were separately monitored with a fluorescence microscope equipped with appropriate sets of filters. When the oligonucleotide was injected, FRET rapidly decreased and it reached plateau in about 1 h (Fig. 5A). The ratio decreased from 2.3 at 1 min to 0.42 at 60 min. This time-dependent decrease of FRET was not observed in the eggs injected with double-labled phosphorothioate, R-S-ODN-F, which is known to be resistant to the digestion by general nucleases (Fig. 5B). These results indicate that the lower extent of FRET in the eggs for the double-labled oligomer with natural phosphodiester

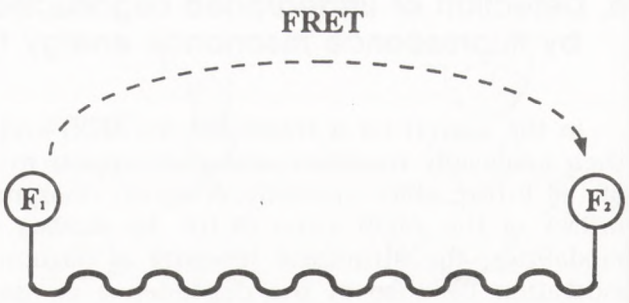

Fig. 4. Detection of undegraded oligonucleotides by fluorescence resonance energy transfer (FRET). A single-stranded oligonucleotide was double-labled with fluorescein (F_1) at its 5'-end and rhodamine X (F_2) at its 3'-end to detect FRET within an undegraded molecule.

linkages was mainly due to the enzymatic digestion of the oligonucleotide. This is the first demonstration of kinetics of oligonucleotide-degradation *in vivo*. Therefore, the FRET technique should be useful for studies *in vitro* and *in vivo* of nucleolytic activity and the integrity of nuclease-sensitive oligonucleotides (47).

6. Activity of Ribozyme *in vivo*

Since the hammerhead and hairpin ribozymes that are generally used in attempts to suppress the expression of specific gene originated from plant viroid or satellite RNAs (6), it seems obvious that ribozymes should be active in plant cells. If ribozymes were to be sufficiently active in other cells, in particular in mammalian cells, ribozymes would be powerful tools for both molecular biological and therapeutic purposes.

Cameron and Jennings were the first to demonstrate that a ribozyme can suppress the expression of a specific gene *in vivo* (48). The target gene for chloramphenicol acetyl transferase (CAT) was introduced into the 3' noncoding region of the gene for luciferase under the control of the SV40 early promoter. A ribozyme-expressing plasmid and the CAT-expressing plasmid were cotransfect monkey cells (COS 1) at a molar ratio 1000:1. The expression of CAT was suppressed to 30% of the control level.

Many similar successful experiments in cells of other organisms have been reported namely, in microorganisms [*E. coli*, yeast (49)], and in cells of an insect (*Drosophila*), a frog (*Xenopus*), the mouse (C127 I cells, NIH 3T3 cells, etc.), and humans (HeLa cells, Jarkat cells, etc.), as well as in a plant [tobacco (50)] (11). Ribozymes appear to be active in any type of cell provided they are properly designed.

It is often argued that the effect of a ribozyme is due to its antisense action and not to its catalytic activity *in vivo* since the RNA tails located on both sides of the catalytic domain of a ribozyme (Fig. 1b) have the antisense sequence (Ys) of the target RNA (Xs) to ensure specificity. Moreover, it is not clear how many free Mg^{2+} ions would be available to ribozymes (it is generally assumed that the intracellular concentration of Mg^{2+} ions is 30 mM and a concentration of Mg^{2+} ions of at least 2 mM is needed for cleavage

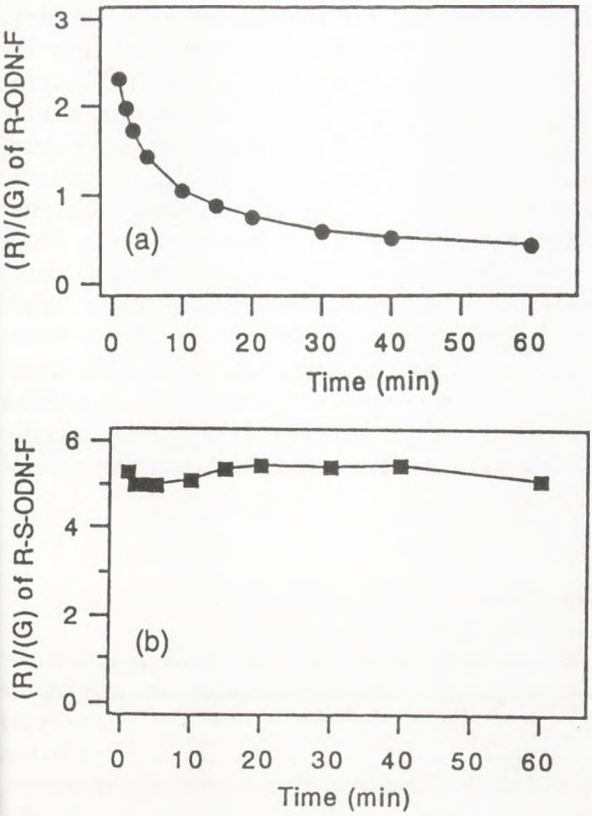

Fig. 5. Time-dependent change of the ratio of the red to the green components of the fluorescence in eggs under the fluorescence microscopy. (a) The ratio of the two color components in eggs injected with R-ODN-F, that has natural phosphodiester linkages, gradually decreased in 60 min. (b) The ratio of the two color components in eggs injected with R-S-ODN-F, that has nuclease-resistant phosphorothioate linkages, was constant over 60 min.

by ribozymes under intracellular conditions). Most Mg^{2+} ions should be bound to nucleotides or to other components such as protein. Such claims of antisense effects arise from the difficulty in detection of cleaved products: the cleaved fragments tend to be degraded rapidly by intracellular nucleases. Some researchers have succeeded in showing the superiority of ribozymes by detecting cleaved products or by comparing the suppressive activity with that of an inactive ribozyme with an altered, and therefore inactive, catalytic sequence (11).

We have also demonstrated that the suppressive ability of ribozymes is due to cleavage of the target RNA rather than to an antisense effect (51). We used an RNA coliphage system because of the ease and rapidity of the evaluation of ribozyme activity *in vivo*. A ribozyme targeting to the A2 gene (for the maturation enzyme) of the RNA coliphage SP and same construct with one change in the catalytic sequence were designed. The latter inactive ribozyme might be expected to form more a stable duplex with the target RNA than the active ribozyme. However, no significant inhibition by the altered construct of the proliferation of phage was observed. This result clearly indicated that the suppressive effect was due to the catalytic activity of the ribozyme rather than to an antisense effect.

The optimal location for maximal ribozyme activity in the cell, namely, the cytoplasm or the nucleus, has not been determined. Recently, Chowrira et al. showed clearly that ribozymes function efficiently at least in the cytoplasm (52). They constructed a *cis*-acting ribozyme-expression plasmid under control of the T7 promoter and transfected T7 RNA polymerase-expressing cells. In this assay system, T7 RNA polymerase is localized in the cytoplasm. Thus, transcription and, therefore, self-processing of the *cis*-acting ribozyme took place in the cytoplasm. Complete self-processing was observed. Thus the cytoplasmic environment can support ribozyme activity. By contrast, since the nucleus contains a large number of RNA-binding proteins, nuclear localization might be disadvantageous for expression of ribozyme activity. However, Tuchihashi et al. showed that some RNA-binding proteins can facilitate ribozyme-catalyzed reactions *in vitro* (30). Moreover, a tRNA (53) or an snRNA (54) embedded ribozyme that is localized in the nucleus can suppress specific gene expression. Thus, the possibility of ribozyme activity in nucleus cannot be excluded.

7. Ribozyme-expression systems

Initial attempts to express ribozymes *in vivo* used the RNA polymerase II (pol II) system that is usually employed for transcription of mRNA or expression of long, antisense RNA. This system generates the cap and poly(A) structures that are essential for stability of RNAs *in vivo*. These structures would be expected to attach to the 5' and 3' ends of ribozyme RNA. However, such modifications allow the binding of ribosomes for translation and might inhibit association with target RNA. Moreover, the promoter is not suitable for production of short RNAs (55). Since, in this system, at least several hundred nucleotides are needed between the promoter and the terminator for effective transcription and termination at correct sites, either extra sequences have generally been added at both ends of the ribozyme sequence or the ribozyme sequence has been inserted into the noncoding region of a stable mRNA, such as CAT mRNA. The long 5' and 3' extra sequences had undesirable effects on cleavage activity (52,56). Thus, 100-fold or 1,000-fold molar excess of ribozyme over the target RNA was required for successful suppression of expression of the target gene *in vivo* (5)

We have developed a new system for expression of ribozymes *in vivo* (and *in vitro*) (57-62). This system consists of a promoter, two *cis*-acting ribozymes and terminator sequences, with a *trans*-acting ribozyme sequence inserted between the two *cis*-acting ribozyme sequences (Fig. 6a). During transcription, the two *cis*-acting ribozymes liberate the short *trans*-acting ribozyme. Using this system, we can exploit any kind of pol II system because of the absence of the of extra 5' and 3' sequences. This system can also be used in the preparation of short RNAs with defined ends for NMR and X-ray analyses (63). We designed five kinds of anti-HIV ribozyme that target different positions in HIV-1. The ribozymes were embedded in the anticodon

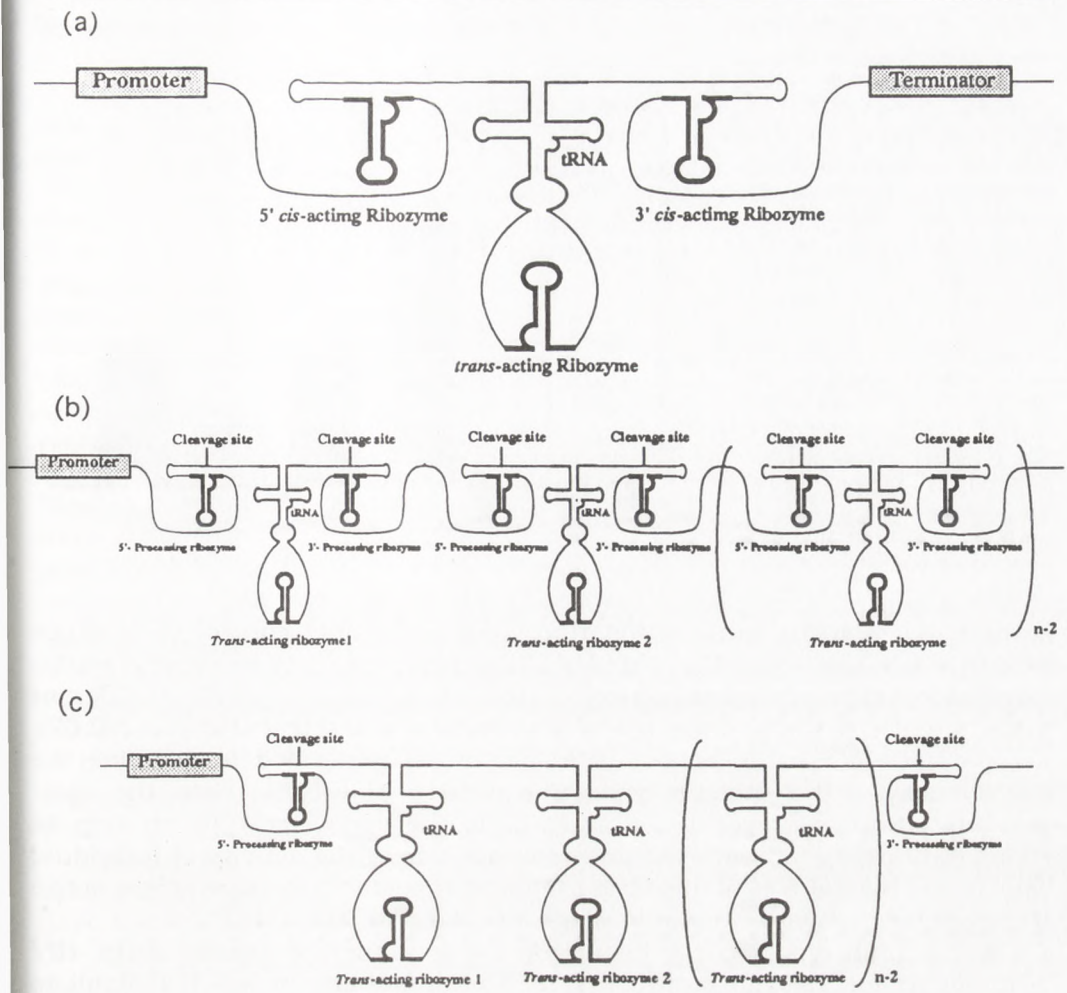

Fig. 6. (a) A novel ribozyme-expression system with two *cis*-acting ribozymes that liberate a tRNA-embedded *trans*-acting ribozyme during transcription. (b,c) Multitarget ribozyme-expression vectors of the shotgun-type (b) and the simply connected type (c).

portion of a tRNA in order to increase the stability *in vivo* (53,60), and then they were introduced between *cis*-acting ribozyme sequences. After connection to a mammalian expression vector under control of the SRa promoter (64), the constructs were used to cotransfect human cultured cells together with the infectious proviral HIV-1 DNA clone pNL 4-3 (65), at a molar ratio of only 1:8 (66). All constructs caused significant inhibition of the production of the HIV-1 p24 antigen (Fig. 7), and two had the strongest inhibitory effect (>80%). This result clearly demonstrates that our system can improve ribozyme activity *in vivo*.

In addition, by combining a ribozyme cassette composed of two *cis*-acting ribozyme sequences and one *trans*-acting ribozyme sequence, we can express

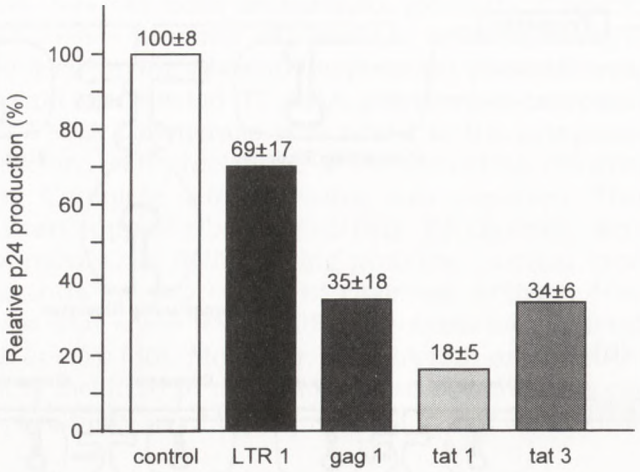

Fig. 7. Replication of HIV-1 in the presence of various ribozyme-expressing plasmids. The transient co-transfection assay was carried out as described previously (31). All constructs (120 ng) were tested by co-transfection of human SW480 cells with infectious proviral HIV-1 DNA (pNL4-3, 40 ng). Plasmid that codes for a ribozyme targeted to *lacZ* (inactive ribozyme for HIV-1; 120 ng) served as the control (100% replication). The columns represent averages of 24 measurements: the average values in % replication are given in numbers above the bars.

several independent *trans*-acting ribozymes under the control of a single promoter (shotgun type; Fig. 6b) (61). There is another way to express multi-target ribozymes: several sequences of ribozymes that are specific to different target sites are simply connected in tandem (connected type; Fig. 6c) (56,67). Comparisons of kinetic behaviors *in vitro* were performed to examine the effectiveness of the shotgun-type strategy (61). At least *in vitro*, the shotgun-type strategy proved to be superior to the connected-type strategy in terms of ribozyme productivity and maintenance of the activity of individual ribozymes. Bertrand et al. also reported that a pentaribozyme was less active than a mixture of five kinds of single ribozyme *in vitro* (56).

The shotgun-type strategy is suitable for gene therapy against AIDS. HIV is infamous for its high mutation rate. This mutability makes it difficult to apply ribozymes to AIDS therapy. Because ribozymes have high specificity for their target sequences, a single point mutation around a cleavage site is sufficient to eliminate effective cleavage. One way to overcome the mutability of HIV would be to use several ribozymes that target different site in HIV simultaneously. Such an idea has been validated using antisense DNA (68). We have already succeeded in cleaving HIV-1 RNA at five different positions simultaneously by using a shotgun-type ribozyme-expression system *in vitro* (61).

Yet, another type of expression system has recently been developed. This system is based on use of RNA polymerase III, which produces small RNAs at levels 10-fold greater than those observed in an RNA polymerase II-based expression system (53). Yu et al. demonstrated the superiority of the pol III system over the pol II system by direct comparisons of suppressive ability *in vivo* (69).

8. Selection of functional RNAs *in vitro* and *in vivo*

Our multiribozyme expression vector by the combination of *cis*- and *trans*-acting ribozymes (Fig. 6b) showed that several ribozymes, each directed against a different target in the HIV genome and acting independently in a "shotgun" manner, markedly increased the efficiency of cleavage of HIV RNA *in vitro* (61). However, the *cis*-acting ribozymes that had trimmed the 5' and 3' ends of each *trans*-acting ribozyme were designed merely to await for degradation by RNases when they were used *in vivo*. Since several *trans*-activator proteins are essential for viral replication of HIV-1, we wondered whether a decoy function could be coupled with the cleavage activity of ribozymes. We therefore introduced the TAR or the RRE sequence into the stem II region of each *cis*-acting ribozyme (70). When the activity of each resulting *cis*-acting ribozyme that had been endowed with the decoy function was examined *in vitro*, it was found to retain almost full trimming activity. Moreover, *cis*-acting ribozymes with either the TAR or the RRE sequence were shown to be able to trap Tat or Rev protein successfully. It is, therefore, possible to endow the stem II region with a specific protein-binding function without the loss of ribozyme function. Thus, *cis*-acting ribozymes, endowed with the decoy function, can first trim the 5' and 3' ends of each *trans*-acting ribozyme and are then still available for trapping *trans*-activator proteins possibly prior to their degradation by RNases when they are to be used *in vivo* (Fig. 8). Furthermore, it is also expected that the reduction in production of HIV RNA that is achieved by sequestering the *trans*-activator proteins might provide the *trans*-acting ribozymes, targeted to HIV RNA, with a better chance of eliminating the remaining HIV RNA.

Ribozymes, the TAR, and the RRE sequences used above are those of the natural origin. In order to improve ribozyme activities, many approaches have been made, including some attempts to select active ribozymes *in vitro* (71-76) and *in vivo* (77). However, as of today, no ribozymes exist that have significantly higher activities than that of the wild-type ribozyme. Nevertheless, the field of selection of catalytic RNAs is growing and we await with great interest the future success. Indeed, we have recently succeeded in selection of an aptamer that binds significantly more tightly to the *trans*-activator protein, Tat (Fig. 8). The K_D of this aptamer was improved up to two orders of magnitude as compared with the K_D of the natural TAR sequence. Since the specificity of this aptamer for Tat is also improved, we can expect an improved decoy function for the selected aptamer sequence.

9. Therapeutic use of ribozymes

The effectiveness of ribozymes against some kinds of cancer and HIV has already been demonstrated in lines of cultured human cells and no significant negative effects on normal cell growth have been reported. Such results encourage efforts to exploit ribozymes for treatment of cancers, AIDS

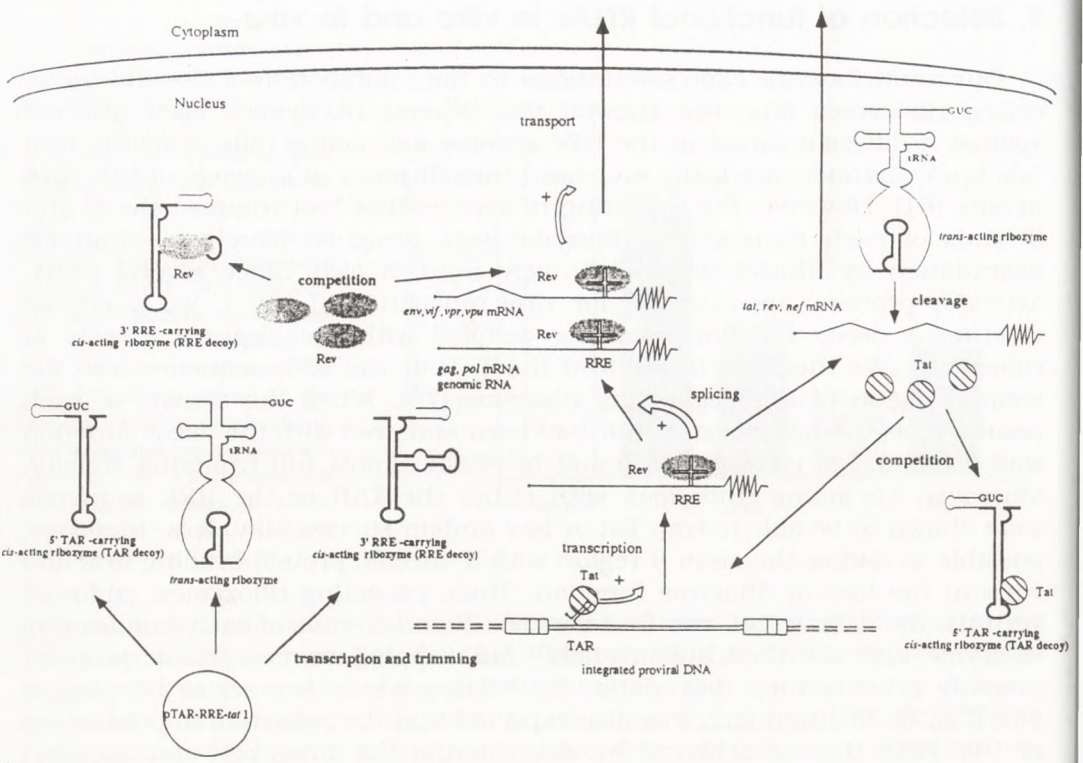

Fig. 8. Hypothetical representation of inhibition of HIV replication by the multifunctional ribozyme expression vector that produces a *trans*-acting ribozyme and *cis*-acting ribozymes with decoy capability. The *cis*-acting ribozymes, endowed with the decoy function by having TAR and RRE sequences, can first trim the 5' and 3' ends of each *trans*-acting ribozyme and are then still available for trapping *trans*-activator proteins. The natural TAR sequence can be replaced by the selected aptamer that is capable of binding to Tat protein with a higher affinity and specificity.

and other human diseases. There are two ways to use ribozymes in therapy: one is to use a chemically synthesized ribozyme as a drug; the other is to express a ribozyme from template DNA (78). The former method as is also true for the antisense strategy, needs a good drug-delivery system suitable for the targeted cells. Moreover, since it is difficult to obtain sufficient amounts of chemically modified, RNase-resistant ribozymes, the use of ribozymes as a drug seems impracticable at present. However, the second way is already being tested. Wong-Staal's group applied to the Recombinant DNA Advisory Committee of the National Institutes of Health (USA) for permission to test a ribozyme strategy for gene therapy of AIDS. Their protocol was accepted in 1993. In their protocol, ribozymes will be used for intracellular immunization against HIV-1. As of January 1996, trials have not yet begun.

10. Conclusion

We have described qualitatively the way in which hammerhead ribozymes function as metalloenzymes. (Quantitative analysis is available in references 26, 41, and 42). Quantitative kinetic analysis revealed that hammerhead ribozymes exploit the general double-metal-ion mechanism of catalysis, wherein metal ions are coordinated directly to the attacking and leaving oxygens (Fig. 3b). Since the catalytic domain captures magnesium ions and magnesium ions can cleave phosphodiester bonds, the extra sequences other than the metal binding site of hammerhead ribozymes can be removed to yield much smaller and highly active hammerhead ribozymes (79). Magnesium ions function also to induce structural changes that are favorable for formation of the active ribozyme complex with its substrate (45). The hammerhead ribozyme is attractive not only as a model system for studies of the mechanism of action of RNA catalysts but also as an agent for controlling gene expression. Ribozymes have been proved to be active in cultured cells at least. We await with great interest the results of initial efforts at ribozyme-based gene therapy.

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Mechanistic Studies of Hammerhead Ribozymes and their Applications *in vivo*

Summary

The hammerhead ribozyme belongs to the class of molecules known as antisense RNAs. However, because of short extra sequences that form the so-called catalytic loop, it can act as an enzyme. Since the catalytic domain captures magnesium ions and magnesium ions can cleave phosphodiester bonds, hammerhead ribozymes are recognized as metalloenzymes. In RNA cleaving reactions catalyzed by protein enzymes, the cleavage of phosphodiester bonds involves acid/base catalysis, with proton transfer occurring in the transition state. When the possibility of such a proton-transfer process was examined, by measuring solvent isotope effects, in reactions catalyzed by hammerhead ribozymes, it became apparent that no proton transfer occurs in the transition state during reactions catalyzed by a hammerhead ribozyme. This and an

additional kinetic analysis, using a natural all-RNA substrate that contains a 5'-thio-leaving group at the cleavage site, revealed that hammerhead ribozymes exploit the general double-metal-ion mechanism of catalysis, with Mg^{2+} ions coordinating directly with the attacking and leaving oxygen moieties. Since the hammerhead ribozyme is one of the smallest RNA enzymes known and has potential as an antiviral agent, thus ribozyme has been extensively investigated for applications *in vivo*. Ribozymes are described that have possible utility as agents against HIV-1.

Key words:
antisense, ribozyme, hammerhead.

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