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Ribozymes: Applications to Functional Analysis and Gene Discovery

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Ribozymes are catalytic RNA molecules that cleave RNAs with high specificity. Since the discovery of these non-protein enzymes, the rapidly developing field of ribozymes has been of particular interest because of the potential utility of ribozymes as tools for reversed genetics. However, despite extensive efforts, the activity of ribozymes *in vivo* has not usually been high enough to achieve the desirable biological effects. Now, by the use of RNA polymerase III (pol III) promoters, the ribozyme activity in cells has been successfully improved by developing efficient transport systems for the transcripts to the cytoplasm. In addition, it is possible to cleave a specific target RNA in cells by using an allosterically controllable ribozyme or an RNA-protein hybrid ribozyme. These ribozymes are potentially applicable to molecular gene therapy and efficient gene discovery systems. Furthermore, the developed pol III expression system is applicable to the expression of small interfering RNAs (siRNAs). The advantage of such ribozymes over siRNAs is the high specificity of the ribozyme that would not cause interferon responses.

Key words: gene discovery, hammerhead ribozyme, maxizyme, RNA-protein hybrid ribozyme.

Hammerhead ribozymes are small and versatile catalytic RNA molecules that cleave RNAs at specific sites (Fig. 1A). The rapidly developing field of RNA catalysts is of current interest not only because of their intrinsic catalytic properties but also because of their potential utility as therapeutic agents and specific regulators of gene expression (*1–8*). However, despite extensive efforts, the activity of ribozymes *in vivo* has not usually been high enough to achieve the desirable biological effects. Unlike *in vitro*, conditions *in vivo* are very complex, and many parameters must be considered. Under certain conditions, the interaction of a ribozyme or its gene with intracellular proteins seems to be significant. Thus, many modifications and improvements are required in the ribozyme expression system as well as methods for introducing ribozymes into cells.

For the successful inactivation of a specific gene by a ribozyme *in vivo*, we have developed an efficient system for the expression of ribozymes, and we have attempted to apply this system for potential gene therapy and functional analysis of genes of interest. In addition, we have developed two novel ribozymes: an allosterically controllable ribozyme and an RNA-protein hybrid ribozyme. The former, termed a maxizyme, has sensor arms that recognize target mRNA sequences, and in the presence of such sequences, the maxizyme forms a cavity that can capture catalytically indispensable Mg^{2+} ions and thereby cleaves the target (*9–13*). The latter, a hybrid ribozyme, which couples the cleavage activity of the hammerhead ribozyme with the unwinding activity of an RNA helicase, can cleave the target mRNA efficiently, regardless of the secondary structure of the target mRNA (*14*, *15*).

1. Methods for the introduction of ribozymes into cells

When ribozymes work inside cells, they must be internalized into individual cells and access the target mRNA. However, cellular uptake of ribozymes and other naked nucleic acids is usually inefficient, due to their charged composition and large molecular size. To overcome this problem, liposomes and charged lipids are commonly used as delivery systems for ribozymes. Complexes of nucleic acids with cationic lipids are usually internalized into the cells by endocytosis.

For application of ribozymes *in vivo*, chemically synthesized ribozymes can be directly administered, or a plasmid vector encoding ribozyme genes can be introduced into cells, where ribozymes can be transcribed by transcriptional factors in the host. Since naked nucleic acids are rapidly degraded by nucleases in cells, especially in the gastrointestinal tract and blood, ribozymes synthesized *in vitro* should be protected by chemical modification, such as thio modification or alkylation at the 2′ position of the ribose ring. Such chemically modified ribozymes are being evaluated in clinical trials (*16*). However, such modifications sometimes result in increased cell toxicity and higher costs for the preparation. In addition, exogenous administration of the modified ribozymes limits the duration of efficacy, even if stability is increased. As an alternative approach, vector-based delivery has attracted attention, because the ribozyme gene can be administered as stable DNA, which

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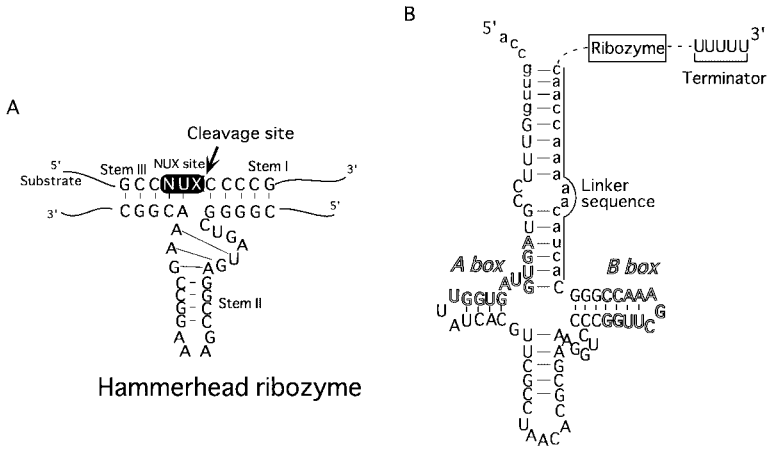

Fig. 1. **(A) Secondary structure of a hammerhead ribozyme.** The hammerhead ribozyme consists of the substrate-binding region (stems I and III) and a catalytic core with a stem-loop II region. When the catalytic core captures the catalytically indispensable Mg^{2+} ions, the cleavage occurs only after the NUX sequence (N, any base; X, A, C, and U). **(B) Secondary structure of a tRNA^{Val}-driven transcript.** In the RNA polymerase III-based transcription system, the promoter is located within the tRNA sequence (indicated as A and B boxes). The ribozyme is linked downstream of the partially modified human tRNA^{Val} through a linker (indicated by underlining).

will then constitutively produce ribozymes inside target cells.

Many viral-based vectors have shown potential as vehicles for *in vivo* gene delivery, including adenovirus, herpes virus, retrovirus, adeno-associated virus, and lentivirus (*8*, *17–23*). Rapid advances in viral vector technology have led to not only improved efficiency for introduction of ribozymes into cells but also the control of transduction, such as tissue-specific transduction. Although various viral vectors are derived from detoxified viruses, their exploitation has been fraught with problems related to production, immunogenicity and safety (*24–26*). Thus, the preparation and purification of many viral vectors are still being improved.

On the other hand, artificial non-viral vectors are currently being developed for non-viral gene and oligonucleotide delivery, since they are more cost-effective and less laborious to produce, and safer to use than the viral vectors. Polymers and cationic lipids are by far the most widely used of the reagents used to enhance intracellular delivery, and recently a wide range of reagents including liposomes, polypeptides and polymeric dendrimers have also been used (*27–30*). However, a critical problem is that the efficiency of introduction by non-viral vectors in specific tissues and organs might be lower than that of viral delivery.

Thus, the development of both viral and non-viral vector–based deliveries is advantageous for introducing ribozymes into cells, depending on the aims. Together with the improvement of delivery technologies, the improvement of intracellular ribozymes is necessary, considering the environment inside the cells.

2. Ribozyme expression system

2.1. Use of pol III system. After introduction of ribozyme-expression vectors into cells, the efficacy of the ribozyme *in vivo* should be influenced by at least five factors: (i) the amount of ribozyme transcripts, (ii) the intracellular stability of ribozymes, (iii) the subcellular localization of the ribozymes, (iv) the activity of the ribozymes, and (v) the accessibility to ribozymes of their target mRNA. Of these factors, (i)–(iii) can be controlled by the choice of an appropriate expression system.

In early studies of ribozyme expression systems, RNA polymerase II (pol II) promoters were widely used as a system for the expression of ribozymes. In this expression system, the cap structure and the poly(A) tail are automatically added at the 5′ and 3′ ends of the transcripts, respectively. Thus, the transcripts are protected from degradation by exonucleases and exported from the nucleus to the cytoplasm, just like an mRNA. However, the pol II expression system that is usually suitable for long RNAs (several hundred to several thousand bases) might not be appropriate for the transcription of short RNAs such as ribozymes. Although the addition of extra sequences around the ribozyme transcript is likely to increase the stability of the transcript in the pol II system, these extra sequences might decrease the activity of the ribozyme by perturbing its higher order structure or by eliciting cellular proteins bound to pol II–driven transcripts.

Thus, RNA polymerase III (pol III) systems seem more attractive for the production of ribozymes than pol II systems. Pol III systems are mainly involved in the transcription of short RNAs such as tRNA (*31*) and U6 snRNA, and their level of transcription is 2–3 orders of magnitude higher than that of pol II systems (*32*). In addition, fewer extra sequences are needed for the transcription. Therefore, pol III promoters should be employed for an ideal system for the expression of ribozymes. Indeed, pol III expression systems based on human tRNA^{met} and U6 promoters have been used for the expression of hammerhead and hairpin ribozymes in cells (*33*).

Among the pol III promoters, we have chosen the human tRNA^{Val} promoter for the expression of ribozymes. In the tRNA promoter-based system, the promoter regions are located within the tRNA sequence. Thus, it is inevitable that a portion of the tRNA becomes incorporated into the ribozyme. In general, initially transcribed tRNAs are processed at the 5′ and 3′ ends, and matured tRNAs are then exported to the cytoplasm from the nucleus. In our tRNA^{Val}-based system, in order to avoid processing at the 3′-end of tRNA, we modified the 3′-end stem sequence to block the release of the ribozyme from the tRNA^{Val} portion, so that the transcript has an extended long sequence including the ribozyme sequence at 3′ end of tRNA^{Val} (Fig. 1B) (hereafter this transcript is referred to as tRNA^{Val}-attached ribozyme). As described below, tRNA^{Val}-attached ribozymes were engineered so that they could be exported to the cytoplasm in mammalian cells

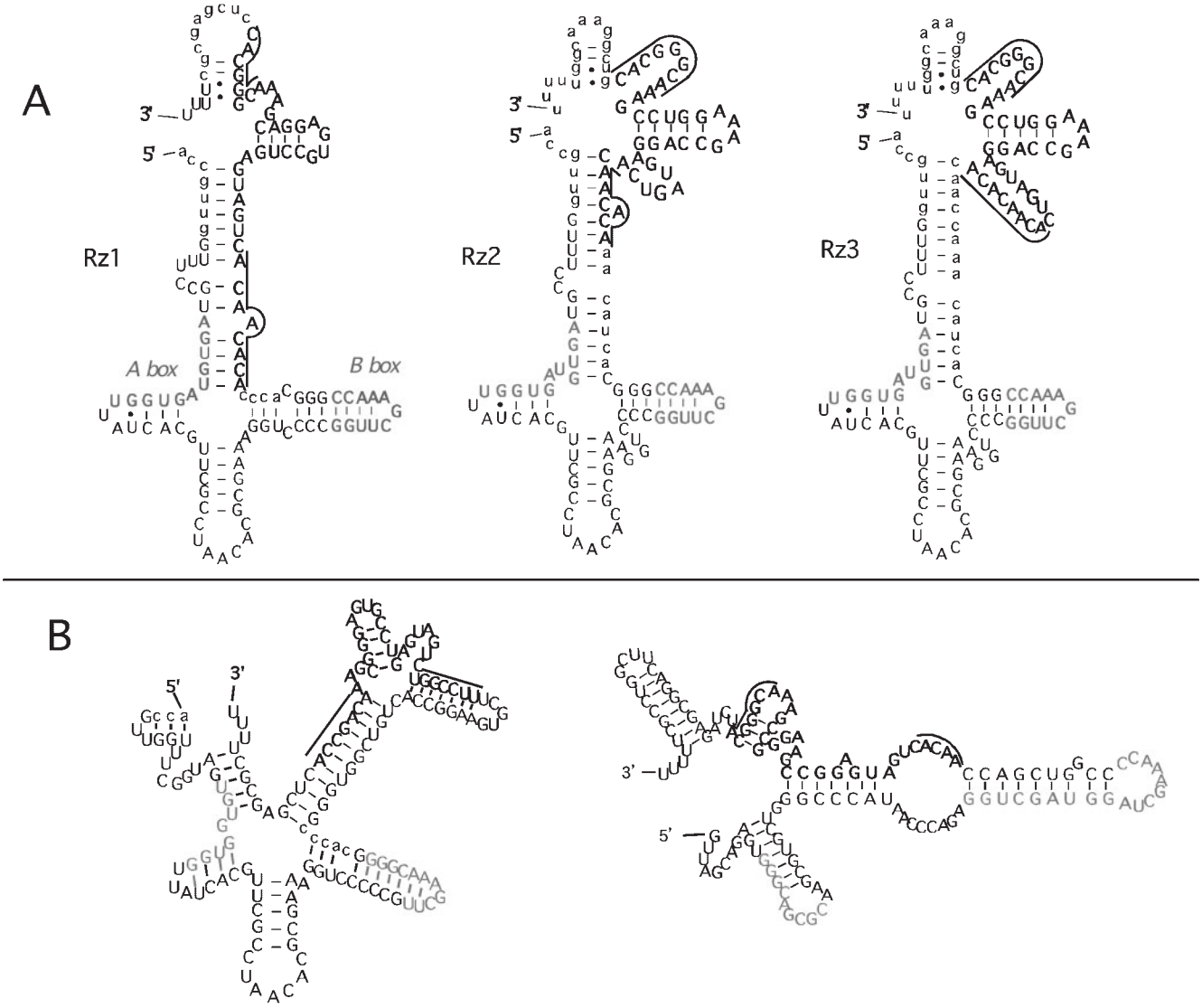

Fig. 2. **(A) Secondary structures of tRNA-attached ribozymes (tRNA-Rzs) that are efficiently exported to the cytoplasm.** Artificial linker sequences are indicated by lower case letters. The ribozyme sequence is shown in boldface and the substrate-binding sites are underlined. **(B) Secondary structures of tRNA-Rzs that accumulate in the nucleus.**

while maintaining a high level of intracellular activities. Moreover, the attached tRNA sequence does not reduce the ribozyme activity, but rather the extra tRNA sequence appears to have a favorable effect on the intracellular activity of ribozymes, most probably by conferring higher resistance to RNases (*34–36*).

Another group has also reported a unique pol III-based system for the expression of ribozymes (*37*, *38*). The dual U6/tRNA^{Lys3} promoter, with U6 and tRNA promoters in tandem, afforded higher levels of transcription than the single tRNA promoter. The dual U6/tRNA^{Lys3}-driven ribozyme exhibited strong activity for the cleavage of cytoplasmic targets.

2.2. Relationship between higher order structure and activity of ribozymes. In our ribozyme expression system, the hammerhead ribozyme is linked downstream of a partially modified sequence of human tRNA^{Val} through a linker (Fig. 1B). We found that the higher order structure of ribozymes strongly affects their intracellular stability. In our system, a small stem-loop structure is added at the 3′ end of the ribozyme to enhance its stability in cells. In addition, the transcript should contain a bulge structure at the double-stranded region corresponding to the linker, in order to block the processing of the pre-tRNA–like transcript and the degradation by nucleases that specifically recognize long double-stranded regions.

In our system, the higher order structure of the transcript is greatly affected by the length of the linker (*34*). Thus, we have investigated the relationship between the higher order structure of ribozyme transcripts and their activity and stability in cells (*34*). We first constructed three different expression systems in which the sequence of the ribozyme targeted against the conserved region in HIV-1 was the same, but the length of linkers was different (Fig. 2A). When the secondary structures of transcripts were predicted by the Zuker method (*39*), the 5′ half of the transcripts including the tRNA portion, but

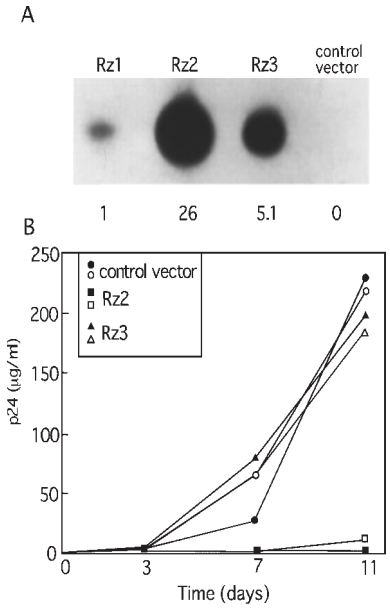

Fig. 3. **Stability of tRNA^{Val} ribozymes in cultured cells.** (A) Steady-state levels of expression of tRNA^{Val}-attached ribozymes. The figure shows Northern blotting analysis with the probes specific for the ribozyme. (B) Inhibitory effects of the tRNA-attached ribozymes on the expression of p24.

not the ribozyme region (indicated by boldface in Fig. 2A), showed closely similar secondary structures to tRNAs.

Owing to catalytic properties of ribozymes, the ease with which they bind to their substrates is an important determinant of their activity. Thus, the degree of freedom and the accessibility of the substrate-binding regions of a ribozyme should substantially determine its activity. In Fig. 2A, ribozymes 1 to 3 (Rz1–Rz3) show different degrees of freedom of the substrate-binding regions (indicated by underlines). As expected, these three ribozymes showed different levels of *in vitro* activity toward a short substrate: Rz3 with the most exposed binding arms had the highest activity (*34*). Next, we examined the intracellular stability of these ribozymes. After transfection of plasmids that encoded each ribozyme, the amounts of ribozymes in the cells were measured by Northern hybridization. Rz2 was 26-fold more stable than Rz1 and 5-fold more stable than Rz3 (Fig. 3A). It remains unclear why these structures dramatically affected the stability of transcripts, because their overall structures are closely similar.

We next evaluated the activity of each ribozyme in cells by using the luciferase reporter assay. The luciferase chosen was a firefly enzyme that catalyzes the chemiluminescent reaction. With the addition of luciferin and the ATP energy source, we could easily and accurately determine the levels of expression of the luciferase by measuring the light emission. In our assay, the ribozyme targeted the 5′ untranslated (5′ UTR) region of the HIV-1 gene fused with the luciferase gene. If the ribozyme cleaves its target efficiently, the level of light emission should be decreased. From this experiment, we confirmed that Rz2, which is the most stable in cells, showed the highest activity of the three ribozymes. The results strongly indicated a correlation between the activity of ribozymes and their intracellular stability. We also investigated the activity of ribozymes toward HIV-1 in tissue culture cells. Cells carrying each ribozyme expression vector were infected with HIV-1, and then the viral protein synthesis was measured as an index of viral replication (Fig. 3B). The result was consistent with the data of luciferase assay. Thus, these results indicate that intracellular stability is one of the most important determinants of ribozyme efficacy (*34*).

2.3. Subcellular localization and ribozyme efficacy. The subcellular localization of the ribozyme after transcription is also an important factor to determine the activity of ribozymes (*40–42*). The target mRNA of the ribozyme is initially transcribed in the nucleus and undergoes maturation via splicing, followed by transport into the cytoplasm, where it is translated into a protein. Because nuclear pre-mRNAs form complexes with heterogeneous nuclear proteins and small nuclear ribonuclear proteins, and interact with various RNA-binding proteins involved in splicing or transport, nuclear pre-mRNAs might be less accessible to ribozymes than cytoplasmic mature mRNAs. Moreover, it is also likely that the stable structure of mRNAs is disrupted by various RNA helicases in the cytoplasm (*14*, *15*). Thus, in order to enhance the efficacy of ribozymes, it is preferable that ribozymes should colocalize with their target mRNA in the cytoplasm.

Our earlier data indicated that tRNA-attached ribozymes with a high level of activity were efficiently exported to the cytoplasm, whereas those with a low level of activity accumulated in the nucleus (*34*, *42*). Thus, we systematically attempted to identify the cellular compartment in which a ribozyme could act effectively. To examine the correlation between the intracellular localization of ribozymes and their activity *in vivo*, we designed several types of ribozymes that targeted the same substrate by using two kinds of promoter. One was the promoter of the gene for tRNA^{Val} described above, and the other was a U6 promoter that transcribes only a portion of the hammerhead ribozyme (*43*, *44*). Thus, we could control the intracellular localization of transcripts in the cytoplasm or in the nucleus using tRNA^{Val} or U6 promoter, respectively.

When the localization of tRNA-driven ribozymes (tRNA-attached ribozymes) and U6-driven ribozymes was determined by Northern blotting analysis after the fractionation of cells, approximately the same levels of expression of transcripts from both promoters were observed, and then tRNA-driven ribozymes localized predominantly in the cytoplasm, whereas U6-driven ribozymes localized in the nucleus. Furthermore, we estimated the activity of these ribozymes in cultured cells. Although both types of ribozyme targeted the identical site and had similar activity *in vitro*, the tRNA-attached ribozymes that were exported to the cytoplasm had strong inhibitory effects, whereas U6-driven ribozymes that remained in the nucleus were completely ineffective. From these results, it became clear that the cytoplasmic localization of ribozymes is a critical determinant of their activity in cells (*42*).

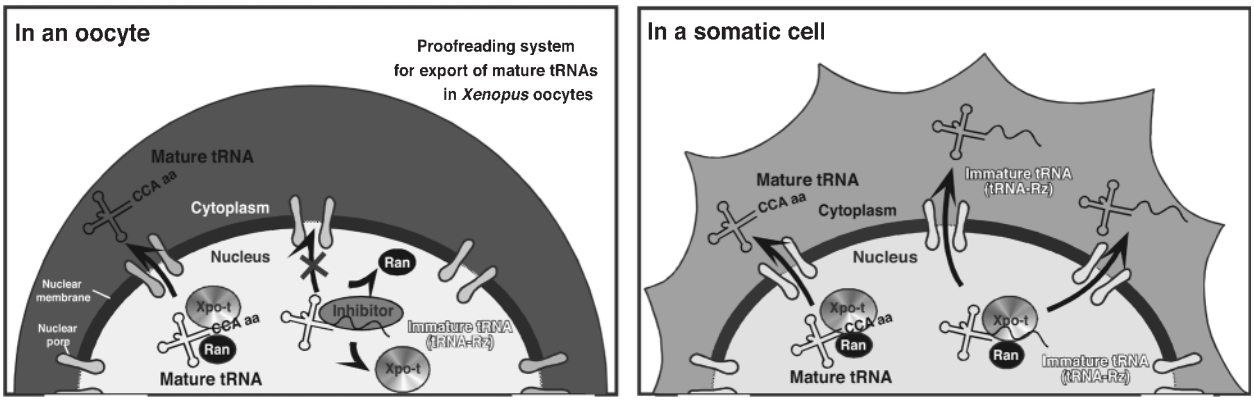

Fig. 4. **Schematic representation of transport to the cytoplasm of mature tRNAs and of tRNA-attached ribozymes.** The original proofreading mechanism has been modified to include the proposed inhibitor-mediated proofreading of tRNA transcripts in *Xenopus* oocytes (left panel). A more general pathway for the export of mature and tRNA-attached ribozymes in somatic cells is presented in the right panel.

2.4. Mechanism of the export of tRNA-attached ribozyme. Although all our $tRNA^{Val}$-attached ribozymes were localized in the cytoplasm, other groups reported that $tRNA^{Met}$-driven ribozymes accumulated in the nucleus (*33*, *41*). The $tRNA^{Met}$-driven ribozyme (Fig. 2B, right) targeted against HIV-1 could not inhibit the expression of the target efficiently (*41*). Comparison of the secondary structures of the $tRNA^{Met}$-driven ribozyme and our $tRNA^{Val}$-driven ribozyme revealed that they were quite different (Fig. 2A and B, right). We further designed another tRNA-driven ribozyme (Fig. 2B, left) and examined its localization. As expected, this tRNA-attached ribozyme remained in the nucleus (*34*).

From these experiments, we clarified that the secondary structure of the tRNA-attached ribozymes is an important determinant of their localization in cells. As shown in Fig. 2A, all $tRNA^{Val}$-attached ribozymes, which are transported to the cytoplasm, form a tRNA-like cloverleaf structure in the 5′ half portion including the tRNA. In contrast, ribozymes remaining in the nucleus do not maintain such cloverleaf structures (Fig. 2B). Thus, we assumed that $tRNA^{Val}$-attached ribozymes with a cloverleaf structure might be exported to the cytoplasm via an export pathway for tRNAs.

In the recent years, rapid progress has been made in efforts to understand the mechanism involved in the export of tRNAs to the cytoplasm (*45–50*). The transport of tRNAs requires a tRNA-binding protein called exportin-t (Xpo-t) and the Ran GTPase, and the rapid transport is accomplished by an interesting mechanism through GTP hydrolysis. According to studies in *Xenopus* oocytes and in yeast, before leaving the nucleus, transcribed pre-tRNAs should be accurately trimmed at both 5′ and 3′ ends and, moreover, CCA must be attached at the processed 3′ end. Only such processed tRNAs can be recognized by Xpo-t (*45–49*). In addition, aminoacylation of the matured tRNAs appears to be critical for their transport from the nucleus to the cytoplasm in *Xenopus* oocytes (*47*) and in yeast (*50*). In *Xenopus* oocytes, immature tRNAs with extra nucleotides at the 3′ end are not recognized by Xpo-t, and thus they can not be exported to the cytoplasm. This phenomenon suggests the existence of a proofreading mechanism in cells, whereby only tRNAs that are usable in the cytoplasm can be exported there. However, our $tRNA^{Val}$-attached ribozymes were efficiently exported to the cytoplasm in mammalian cells (*9–11*, *36*, *42*, *51–54*), even though they could be considered equivalent in form to certain immature tRNAs because of the extra sequences at their 3′ ends. Thus, we investigated the discrepancy between the reported observations in *Xenopus* oocytes that led to the proposal of the existence of a proofreading mechanism and our own observations of the efficient export of $tRNA^{Val}$-attached ribozymes (*55*).

We first considered the possibility of an alternative pathway for the export of tRNAs. In yeast, the fact that genetic mutation of los-1p, a yeast homologue of Xpo-t, did not affect cell survival suggested the existence of additional pathways for tRNA export. However, contrary to our expectation, we found that Xpo-t could recognize $tRNA^{Val}$-attached ribozymes in a similar manner to the tRNA, and Xpo-t was likely to be involved in the transport of $tRNA^{Val}$-attached ribozymes in somatic cells (*54*, *55*). In contrast, a tRNA-attached ribozyme was not exported to the cytoplasm in *Xenopus* oocytes, as predicted by the proofreading hypothesis. Further investigation revealed that the Xpo-t/RanGTP complex did not interact with tRNA-attached ribozymes in oocytes, even though such an interaction could be observed *in vitro* and, more importantly, in several lines of somatic cells. These findings hinted at the presence of inhibitor(s) in *Xenopus* oocytes rather than the involvement of an alternative pathway in somatic cells. In fact, a nuclear extract from *Xenopus* oocytes strongly inhibited the export of tRNA-attached ribozymes in somatic cells, suggesting the presence of a strong inhibitor(s) in oocytes (*55*).

It seems likely that the export of tRNAs in oocytes is subject to a special kind of regulation. Moreover, the proofreading mechanism in *Xenopus* oocytes seems to be involved with a specific inhibitor(s) that might recognize immature tRNAs including tRNA-attached ribozyme specifically. In somatic cells, when the linker and the ribozyme sequence were appropriately adjusted, a tRNA-attached ribozyme seems to be recognized by Xpo-t and then exported to the cytoplasm (Fig. 4).

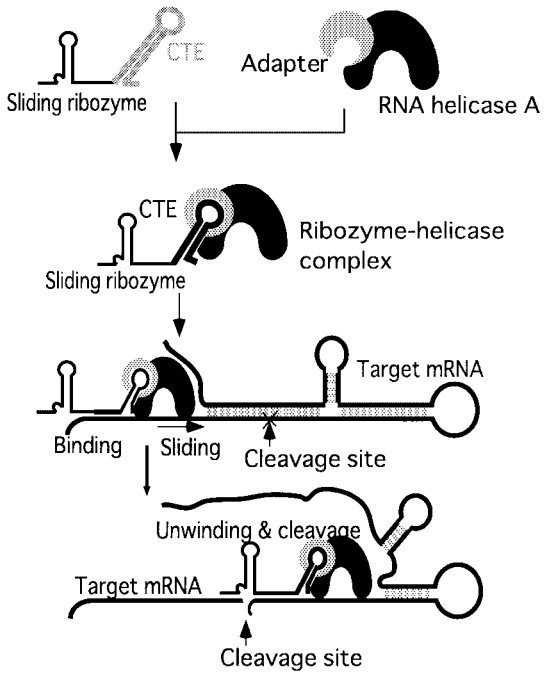

Fig. 5. **Schematic model of how a ribozyme with a CTE motif (CTE-Rz) coupled to an RNA helicase cleaves a hidden cleavage site by unwinding the local secondary structure of target mRNA.**

3. Accessibility of target mRNA to ribozymes

Significant activity of a ribozyme inside cells depends on its high-level expression, intracellular stability, and efficient export to the cytoplasm. In addition to these parameters, the accessibility of the ribozyme's target should be also considered, because the rate-limiting step for the cleavage of substrates *in vivo* seems to be the association/annealing of the ribozyme with its target (*42*).

To enhance such accessibility, computer-generated predictions of secondary structure are typically used to identify target sites that are most likely to have an open conformation (*39*). However, these predictions are often inaccurate because of unpredictable RNA-protein interactions that change the structure of RNA in cells. To circumvent this limitation, a systematic approach involved in searching for huge numbers of candidate sites for antisense molecules has been used (*56*, *57*). Another approach involved in oligonucleotide-directed RNaseH cleavage of target mRNA in cell extracts has also been used. It was demonstrated that the efficacy of RNaseH cleavage elicited by oligonucleotides well correlated with that of ribozyme-induced cleavage. Thus, accessible sites for ribozymes were often selected using random oligonucleotide libraries (*58–60*). A random ribozyme library was also employed to identify ribozymes that cleave the target mRNA efficiently (*61*). Although these approaches seemed to be sophisticated and useful, we sought a simpler strategy. We tried to create a unique ribozyme that would be able to access any chosen target site regardless of its secondary structure (*14*).

3.1. RNA-protein hybrid ribozymes. We reasoned that it would be useful to design a ribozyme that would recruit a protein that unwinds any interfering secondary structures, thereby making any site accessible to the ribozyme. To create such a ribozyme, we tried to link a ribozyme to an RNA helicase, a protein defined as having nonspecific RNA binding, sliding, and unwinding activities (*62–65*). For recruiting the RNA helicase to the ribozyme, we first employed the constitutive transport element (CTE), an RNA motif that could interact with an RNA helicase A *in vitro* and *in vivo* (*66–74*). Then, we hypothesized that an RNA helicase coupled to a ribozyme might efficiently guide the ribozyme to its target site by resolving any inhibitory mRNA structures, thereby leading to efficient substrate cleavage (*14*, *75*).

Figure 5 shows schematic representation of how a CTE-ribozyme coupled to an RNA helicase can cleave a hidden target site by unwinding the local secondary structure. To evaluate the expected enhanced ribozyme effect, we designed a vector that expresses the ribozyme with the CTE from the $tRNA^{Val}$ promoter. As the target of the ribozyme, we chose the TAR region within the long terminal repeat (LTR) of HIV-1 (*11*). Since the conserved TAR region is essential for replication of HIV-1, this region might be an ideal target for gene therapy against HIV-1. Because of its stable stem form, however, it seems difficult for ribozymes to access the TAR region efficiently. If the RNA helicase attached ribozyme through the CTE could disrupt the stem structure and efficiently cleave the TAR sequence, the ribozyme might be an effective drug against HIV-1 regardless of rapid mutations of HIV-1.

Thus, we designed CTE-connected and unconnected ribozymes targeted against the TAR region, as shown in Fig. 6A. At the same time, we established a HeLa cell line that stably expresses the LTR of HIV-1 fused with a luciferase gene for evaluating the inhibitory effect of ribozymes. Figure 6B shows the levels of suppression of LTR-driven luciferase by CTE-ribozymes. As expected, TAR Rz4 and TAR Rz5 without the CTE (non-CTE ribozymes) were unable to inhibit the activity of the luciferase reporter, because they could not access their target sites efficiently. Surprisingly, on the other hand, when the CTE was attached to these ribozymes, their inhibitory effects were significantly enhanced (Fig. 6B, lanes 11 and 13), resulting in about 80% reduction of the reporter activity. Furthermore, these CTE-coupled ribozymes showed higher activity than non-CTE ribozyme designed to target "open" sites (TAR Rz1, LTR Rz2, Luc Rz3). Importantly, TAR CTE-Rz4 and CTE-Rz5 could inhibit the expression of the reporter gene as well as TAR CTE-Rz1, LTR CTE-Rz2, and Luc CTE-Rz3. These results suggested that the attachment of CTE enables all ribozymes to access and cleave their target sites efficiently (*14*).

To examine the general applicability of the CTE-ribozyme, we tested other CTE-ribozymes targeted against several endogenous genes, such as mouse procaspase-3 (CPP 3). The cleavage activity of these ribozymes, as expected, was significantly enhanced, compared to that of parental non-CTE ribozymes (*14*). Thus, we demonstrated that CTE-ribozymes have specificity, significant activity, and general utility. All of the CTE-ribozymes showed robust activity in cell culture, even if the parental ribozymes without CTE showed weaker activity (*76*). Therefore, we believe that such hybrid

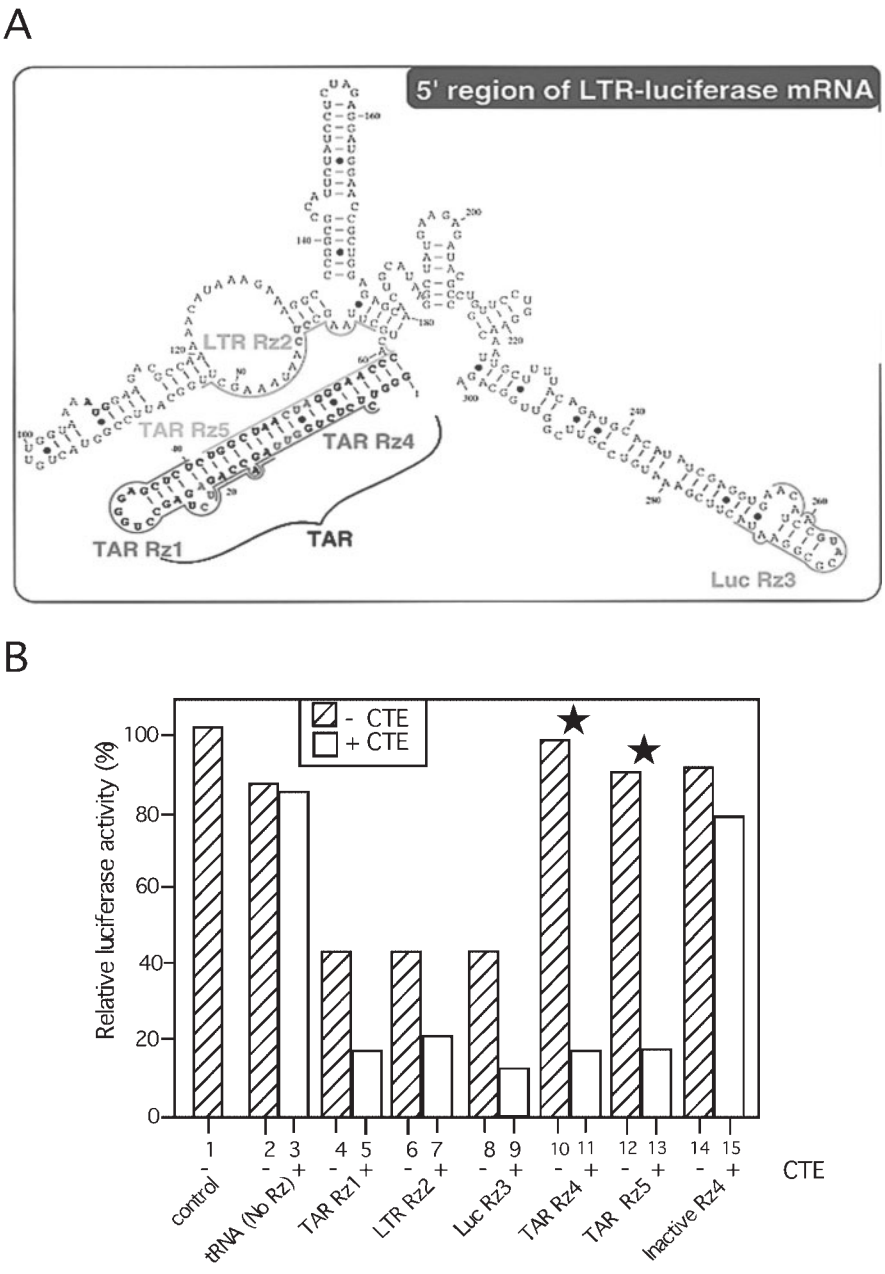

Fig. 6. **(A) The secondary structure (predicted by MulFold) of the 5′ region of long terminal repeat (LTR)-luciferase mRNA and target sequences for each ribozyme. (B) Suppression of LTR-driven luciferase activity by CTE-Rzs. Stars indicate results obtained with Rz targeted to relatively inaccessible sites of the TAR.** Luciferase activity as an indicator of Rz activity is reported as a percentage of the Tat-only control. In all cases, the CTE-Rz always had significantly greater activity than the non-CTE-Rz.

ribozyme-based technology is broadly applicable due to the ease of design and significant activity in cells. In addition, CTE-ribozymes are suitable for a wide range of applications, as described below. As also described below, we later used an alternative motif, the poly(A) sequence, which can recruit a different kind of RNA helicase, eIF4AI, in mammalian cells (*75*).

3.2. Identification of functional genes by use of hybrid ribozymes. Based on the improved intracellular activities of the hybrid ribozymes, we next attempted to identify genes associated with specific phenotypes in cells by using the highly active ribozymes with randomized binding arms. Independently, the Wong-Staal, Barber and Taira laboratories developed a novel gene discovery system using randomized ribozyme libraries that were introduced into cells and produced a phenotypic change (*75*, *77–89*). As reported with hairpin ribozymes (*77–80*), randomization of a ribozyme's binding arms allows easy identification of genes of interest. Screening by use of randomized ribozymes that promote anchorage-independent cell growth identified *hPPAN*, the human homolog of the *Drosophila* ppan gene, and the murine telomerase reverse transcriptase (*mTERT*) gene, as tumor suppressors (*77*, *78*). Another approach using random ribozyme libraries identified *ID4* as a regulator of BRCA1 expression (*79*).

In our case, we identified functional genes by using the hybrid hammerhead ribozyme, which coupled cleavage activity with the unwinding activity of an endogenous RNA helicase (*81*, *82*). In this study, we created novel hybrid ribozymes by attaching a poly(A) tail (a stretch of 60 As) to the 3′ end of a tRNA-attached ribozyme (Rz-A60) instead of the CTE (*81*). The poly(A) sequence is known to interact with an endogenous RNA helicase,

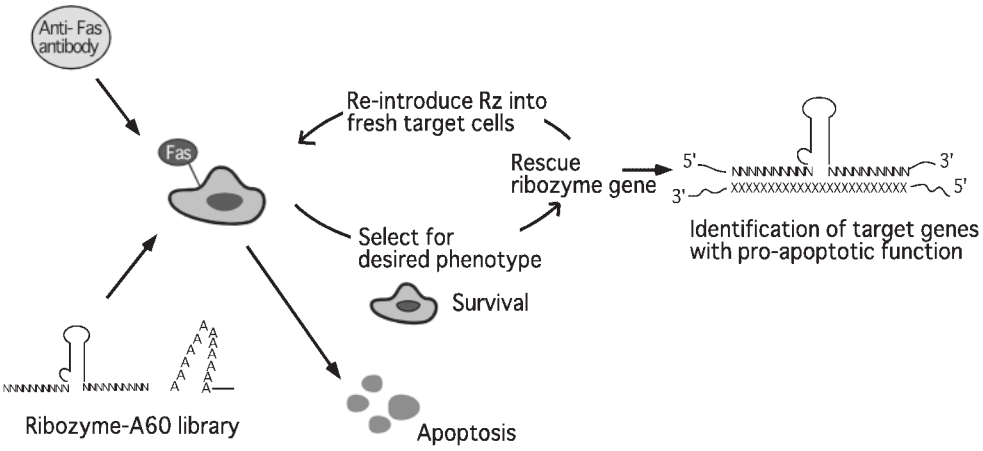

Fig. 7. **Schematic diagram of the gene discovery system for identification of genes involved in the Fas-induced apoptosis by a poly(A)-connected hybrid-Rz library.**

elF4AI, via interactions with poly(A)-binding protein (PABP) and PABP-interacting protein-1 (PAIP). We expected that the poly(A)-mediated complex would unwind the RNA duplex of the substrate efficiently, and then cleave inaccessible target sites. By introducing the library of hybrid ribozymes with randomized binding arms into cells, any cell might show phenotypic changes. Then, the target genes involved in such phenotypic changes can be identified by sequencing of the specific ribozyme clones (*81*, *82*).

For example, using randomized Rz-A60–expression libraries, we established a novel system for screening the genes involved in a signal pathway for Fas-induced apoptosis in HeLa-Fas cells (Fig. 7). In this system, retroviral vectors carrying Rz-A60 with 10 nt randomized sequences in each substrate-binding arm were employed to introduce the libraries into HeLa-Fas cells. Cells were then treated with the Fas-specific antibodies, and survivng cells were harvested for the subsequent isolation of ribozymes. Sequencing of the randomized region of the isolated Rz-A60 enabled us to rapidly identify genes that are responsible for the Fas-induced apoptotic pathway. By this screening, we identified many interesting genes, such as *FADD*, *caspase-8*, *caspase-9* and *caspase-3*. When ribozymes without the poly(A) tail were used for the screening, we could not identify genes such *FADD* and *caspase-8*. This result strongly suggested that the hybrid Rz enhanced the successful screening in our gene discovery system. Indeed, with our gene discovery system, we identified many genes (*82*) involved in other apoptotic pathways (*75*, *83*, *84*), tumor invasion (*85*, *86*), and Alzheimer's disease (*87*, *88*). Importantly, we also identified novel non-coding RNAs by use of our gene discovery system (*89*). With the revelation of the human genome, this unique technology becomes extremely valuable for the identification of important genes.

4. Maxizymes, allosterically controllable ribozymes

Next, we describe a new type of ribozyme that has been developed through the studies of the shortening hammerhead ribozyme. We succeeded in creating an allosteric ribozyme, termed a maxizyme, which functions as a dimer with significant specificity and activity both *in vitro* and *in vivo* (*9–13*). This allosteric ribozyme could be defined as a biosensor *in vitro* and *in vivo* (see below).

First, we created a hammerhead ribozyme without the stem II region and evaluated its activity *in vitro*. Initially, the shortened (minimized) ribozyme was termed a "minizyme," but this term has the negative connotation of a ribozyme with minimal activity. Our minizymes can function as dimers, and they have extremely high activity *in vitro* and *in vivo* (*9–11*). Thus, we re-named the new dimeric minizyme "maxizyme" (minimized, active, x-shaped [functions as a dimer], and intelligent [allosterically controllable] ribozyme).

4.1. Shortened hammerhead ribozymes which function as dimers. The cleavage of substrate RNA by the ribozyme usually requires a divalent metal ion such as magnesium (*90–101*). A catalytic domain of the hammerhead ribozyme captures the catalytically indispensable Mg^{2+} ions. The precise control of conformational change of the ribozyme, in the presence of Mg^{2+} ions, is needed to create an allosteric ribozyme.

Since the downsizing of the hammerhead ribozyme is desirable for medical applications, researchers have generated the compact size of ribozyme by replacing the stem-loop II region with a short linker (*102–105*). Whereas most minizymes showed quite low activity compared to the parental ribozymes, we were able to create a shortened ribozyme lacking the stem-loop II region that had high activity equivalent to the parental one (*105*). Further kinetic and NMR analyses revealed that this shortened ribozyme exhibited extremely high activity as a dimer (Fig. 8A) (*9*, *106*).

Although we initially called this shortened ribozyme a "dimeric minizyme," we later renamed this molecule "maxizyme" (*9–13*). As shown in Fig. 8B, we also designed a heterodimeric maxizyme using two different monomers, maxizyme left (MzL) and maxizyme right (MzR) (*10*, *11*, *106*). Since this maxizyme has two substrate-binding arms, it can recognize two sites on the same target RNA simultaneously. This unique feature of the maxizyme led us to create an allosteric enzyme with sensor function, as described below.

4.2. Design of an allosterically controllable maxizyme. As we first stated that ribozymes can target RNAs at specific sites, certain minimum sequences are required for efficient cleavage. The cleavage of a substrate occurs immediately after the NUX sequence (where N is any base and X is A, C, or U) within the target (*92*, *107*). Therefore, in some cases, the cleavable tri-

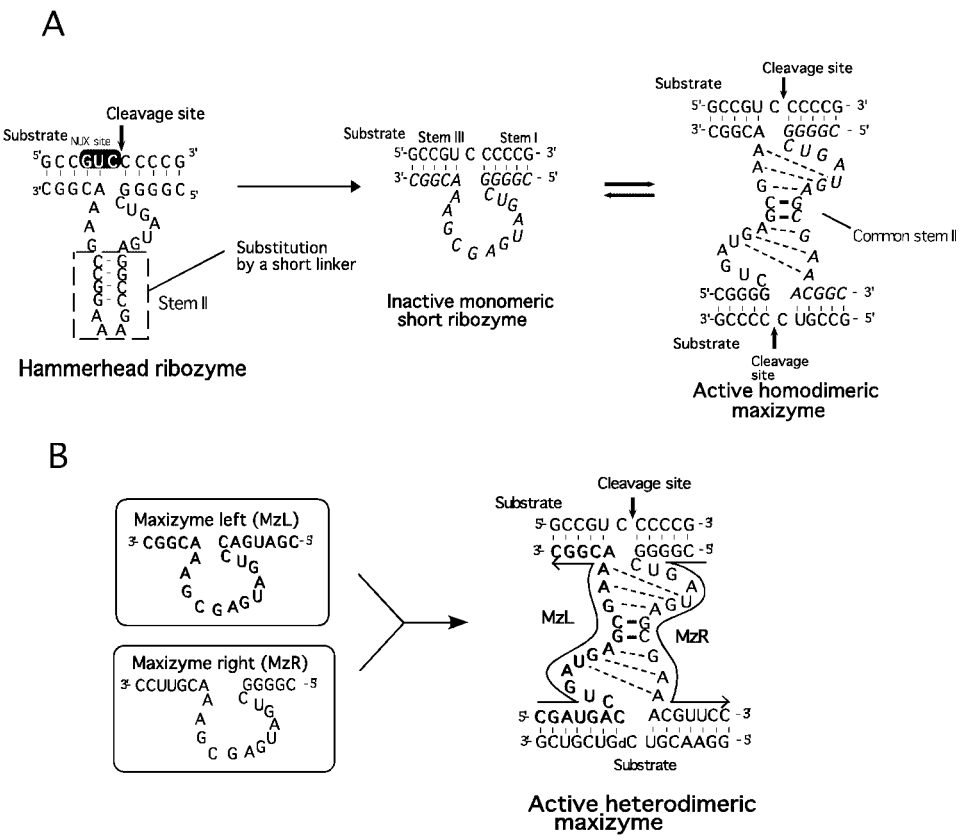

Fig. 8. **(A) Development of an allosterically controllable maxizyme.** Secondary structures of a parental hammerhead ribozyme (left), an inactive monomeric short ribozyme (middle) and an active (homo) dimeric maxizyme (right). A monomeric short ribozyme termed a minizyme was designed by deleting a stem-loop II region from a hammerhead ribozyme. **(B) The heterodimer (MzL and MzR) can generate two different binding sites: one is complementary to the sequence of interest [activator or inhibitor], and the other is complementary to a cleavable sequence.**

plet sequences may not be located at a suitable position on the target. For example, this problem occurs with certain abnormal chimeric mRNAs. Since chimeric mRNA is usually generated via chromosomal translocation, the two halves of the fused mRNA are derived from different genes. Such chromosomal translocation is often involved in the pathogenesis of disease. A well-known example is the Philadelphia chromosome, which causes chronic myelogenous leukemia (CML) (*108*, *109*). The Philadelphia chromosome occurs as a result of reciprocal translocation involving the *BCR* and *ABL* genes, resulting in a fused mRNA. Being tumor-specific and pathogenetically important, fused mRNAs are ideal targets for nucleic acid-based therapeutics as a paradigm (*110*). Nevertheless, because of the absence of NUX sequences near the chimeric junction, a wild-type hammerhead ribozyme could not exclusively target the chimeric mRNA (*10*, *99*, *111*). Since both *BCR* and *ABL* genes are normally important for cell survival, it is essential to destroy only the abnormal chimeric mRNA, without affecting the normal mRNA.

Although attempts were made to cleave only chimeric mRNAs using wild-type hammerhead ribozymes, these were unsuccessful (*112*, *113*). As mentioned above, the maxizyme can bind to two different target sites. Thus, if the maxizyme can simultaneously recognize a junction site by one of its arms and a distant NUX site by the other, it should be possible for the maxizyme to cleave only the chimeric mRNA. Through repeated trial and error, we established an ideal maxizyme that has two independently functional arms. One arm can only bind the substrate as the "eye (sensor)" to discriminate the chimeric mRNA from normal mRNA, and the other arm serves as the "scissor" to actually cleave the target (Fig. 9B, left).

In designing an allosteric maxizyme, it is important that the dimer can act as a molecular switch. In other words, only when the junction sequence is present the maxizyme should take an active conformation by binding to the junction, followed by cleaving the NUX sequence. The precise switching should be controlled through the pairing of bases between the MzL and MzR (Fig. 9A). If the base pairing is too stable, the dimer will take the active conformation even in the absence of a substrate. In contrast, if the base paring is less stable, the dimer will not take the active conformation even if substrate RNA is present. Thus, the precise control of the stability of the base paring is a key point in generating the allosterically controllable maxizyme.

Figure 9A shows a schematic representation of the maxizyme with active and inactive conformations. The active conformation as the dimer can be formed only when the two arms of the maxizyme bind correctly to the two sites on the chimeric mRNA (Fig. 9A, left, and Fig. 9B, left). In contrast, in the presence of only normal *ABL* mRNA, the maxizyme takes an inactive conformation by changing the structure of the central core (Fig. 9A, right). The maxizyme with the inactive conformation cannot cleave its substrates, because it cannot capture the magnesium ions that are essential for its cleavage activity. When chemically synthesized maxizymes were evaluated *in vitro* for specificity to their substrates, they were found to specifically cleave only the chimeric *BCR*/*ABL* mRNA, without damaging the normal *ABL* mRNA.

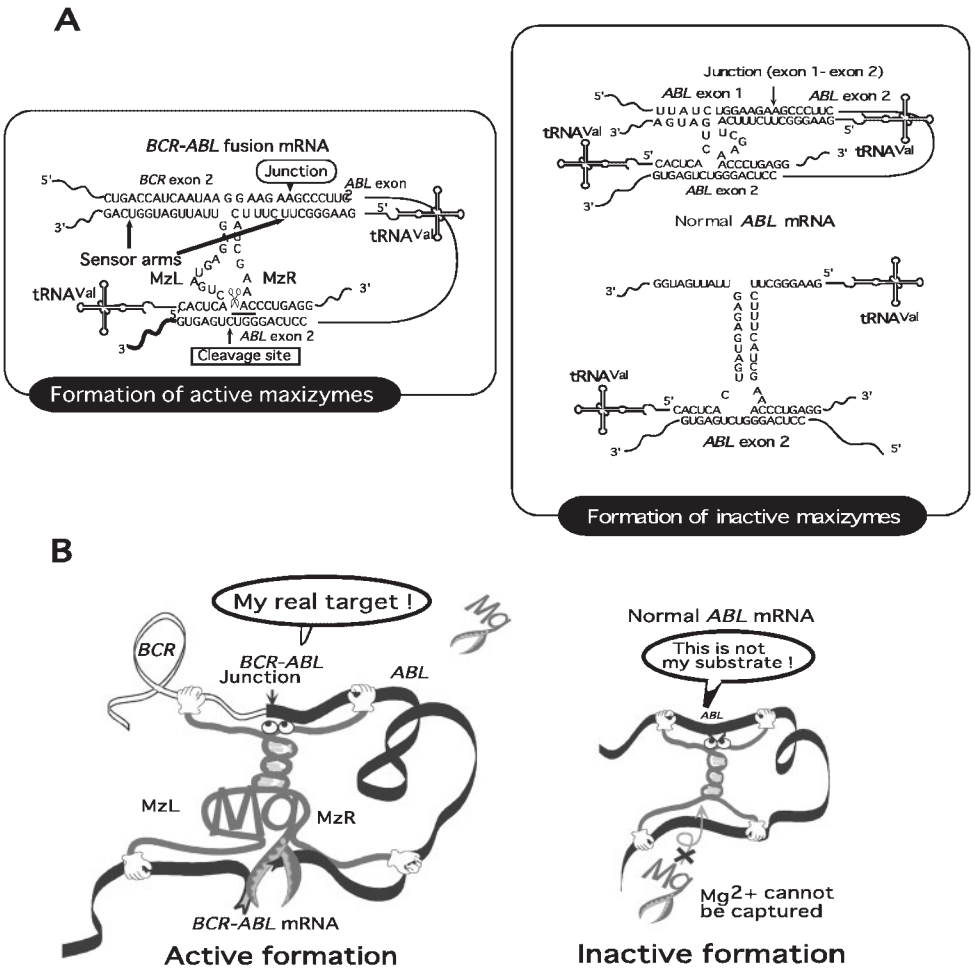

Fig. 9. **(A) Formation of active or inactive maxizymes regulated by specific effector sequences.** The heterodimer (MzL and MzR) can generate two different binding sites: one is complementary to the sequence of interest [activator or inhibitor], and the other is complementary to a cleavable sequence. To exhibit a strict substrate-specificity, maxizymes should take an active conformation only in the presence of the abnormal *BCR–ABL* junction (left), but not in the presence of only normal *ABL* mRNA or in the absence of the *BCR–ABL* junction (right). **(B) Maxizymes can discriminate limited differences among various transcripts, and cleave specifically the correct target in each case.**

We also evaluated the efficacy of the maxizyme in cultured mammalian cells. For producing large amounts of maxizyme in cells, the $tRNA^{Val}$-promoter was again employed for its expression. The high level of expression ensures efficient dimerization. When maxizimes were introduced into cells derived from patients with CML, they showed extremely strong activity compared to parental wild-type ribozymes (*10*). Moreover, as expected, maxizymes inhibited the expression of the *BCR-ABL* mRNA specifically, without affecting that of normal *ABL* mRNA.

Thus, we succeeded in creating an artificial allosteric enzyme, maxizyme, that exhibits high specificity and high activity only in the presence of the oncogenic chimeric mRNA (*10*, *12*).

4.3. Oncogene inactivation in a mouse model. We further examined the anti-tumor effect of the maxizyme in animal models (*12*). To introduce the maxizyme into leukaemic cells, we used a retroviral vector that encoded the two components of the maxizyme under the control of the $tRNA^{Val}$ promoter in tandem. CML cell lines (BV173) that had been transduced with either the maxizyme gene or a negative control gene were independently injected into the tail veins of mice. All of the mice injected with control BV173 cells died of diffuse leukaemia, confirmed at necropsy, 6–13 weeks afterwards (median survival time, 9 weeks), whereas mice injected with maxizyme transduced BV173 cells remained healthy (Fig. 10). The results indicated that the maxizyme introduced by the retroviral vector could be produced at sufficient concentrations to support the dimerization *in vivo*. Moreover, the maxizyme successfully functioned in animals.

At present, kinase inhibitor (*114*) or allogeneic transplantation is employed for the effective therapy of this type of leukemia. Only half of the patients on average would be eligible for the transplantation because of limited donor availability and age restrictions. Our results raise the possibility that the maxizyme could be useful for purging bone marrow in cases of CML treated by autologous transplantation, when it would presumably reduce the incidence of relapse by decreasing the tumorigenicity of contaminating CML cells in the transplant (*12*).

4.4. Generality of the maxizyme technology. The maxizyme technology is not limited to the disruption of the abnormal chimeric gene in CML disease. Abnormal chimeric genes generated from reciprocal chromosomal translocation have frequently been observed in several leukemia diseases. Maxizymes have successfully cleaved only abnormal targets that lack a NUX cleavage site near the junction in cases of acute lymphoblastic leukemia (ALL) and acute promyelocytic leukemia (APL) without damaging normal genes (*13*). Abnormal chimeric mRNAs are also generated as results of mis-splicings. A max-

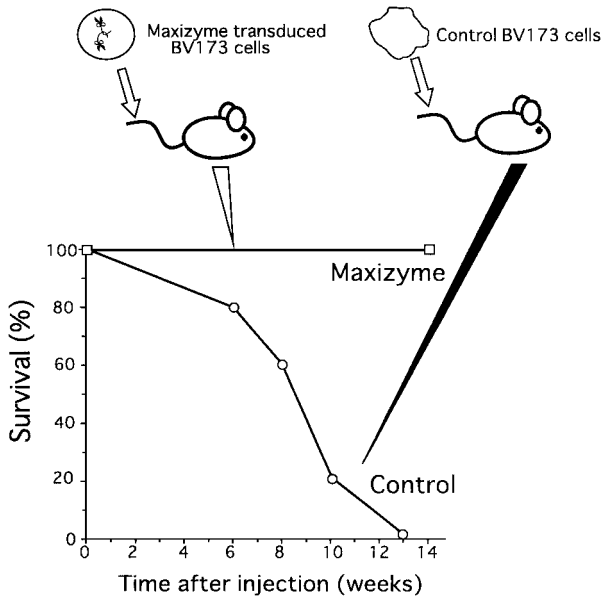

Fig. 10. **Anti-tumor effects of the maxizyme in a murine model of chronic myelogenous leukaemia (CML).** The survival of animals was monitored daily for more than 20 weeks after inoculation. All control mice died within 13 weeks, whereas maxizyme-treated mice remained disease-free for the entire period of the investigation.

izyme designed against each splicing variant could discriminate the target specifically by its allosteric function. Indeed, we have already constructed various maxizymes that target different chimeric genes, and these maxizymes showed significant activity and high specificificity (*10*, *12*). Thus, maxizymes should be considered to be powerful gene-inactivating agents with allosteric functions for cleaving any type of chimeric mRNA. Importantly, to our knowledge, the maxizyme is the first artificial allosteric enzyme whose activity was demonstrated at the animal level, extending its potential utility into the medical area (*12*, *115*). It should be mentioned, however, that this kind of chimeric genes could be also destroyed by siRNAs (*116*, *117*).

5. Summary and prospects

Ribozymes have become a very useful tool for the suppression of gene expression *in vivo*. Thus, ribozymes are usually employed for functional analysis of genes, and a number of ribozyme-based drugs have entered clinical trials (*118*, *119*).

In recent years, small interfering RNA (siRNA)-based technology has been progressively developing as well as the ribozyme technology. siRNA is a 21–23-nt RNA duplex with 2- or 3-nt overhanging at the 3′ ends. These small RNAs can induce a sequence-dependent degradation of a cognate mRNA in the mechanism of RNA interference (RNAi). Several applications of RNAi in mammals hold the promise of systematic analysis of gene expression and therapeutic gene silencing (*120*).

Recently, we noticed that our systems for the expression of ribozymes were applicable to the expression of siRNAs. Due to the small length of siRNAs, pol III systems were proven to be suitable for the production of siRNAs. Moreover, pol III systems would promise high level of transcription. These advantages make it possible to use the pol III systems to synthesize small RNAs *in vivo*.

A number of groups have developed plasmid-based vectors for the expression of siRNAs using several pol III promoters (*121–127*). Among these pol III promoters, U6 and H1 promoters have been predominantly employed. As mentioned previously, the $tRNA^{Val}$-based system is very useful for the expression of ribozymes. The system allows not only high production of transcripts but also selective cytoplasmic localization when the transcript takes an appropriate conformation. Since RNAi in mammalian cells could occur in the cytoplasm restrictively (*128*), it is likely that the $tRNA^{Val}$-based system is also suitable for production of siRNAs. In fact, when short hairpin RNAs (shRNAs) that could be processed to siRNA in cells were produced from $tRNA^{Val}$ promoter, the $tRNA^{Val}$-attached shRNAs were efficiently transported to the cytoplasm, where they effectively induced RNAi-mediated gene silencing (*129*). Thus, a tRNA-based system should also be useful for the expression of siRNAs.

There is no doubt that siRNAs are potentially a powerful tool for the functional studies of genes, and might also be useful as future therapeutic agents (*130*). Now, we can choose two versatile RNAs, ribozymes and siRNAs, for use as gene knockdown tools. Since siRNAs are involved in cellular mechanisms, there exist many proteins that assist the cleavage of the target RNA, whereas the ribozyme cannot depend on intracellular factors. Thus, naturally, the siRNA is more effective than the ribozyme. However, the advantage of the ribozyme is the absence of any interferon responses and, thus, we can clearly detect only the specific effects, despite its lower activity than siRNA (*131–133*). In conclusion, for functional analysis of genes of interest, it is advisable to use both siRNA and ribozyme technologies in parallel. Indeed, by this combination, we could identify important genes that are involved in siRNA pathways.

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