Specificity of novel allosterically *trans*- and *cis*-activated connected maxizymes that are designed to suppress *BCR-ABL* expression

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Abstract **Chronic myelogenous leukemia (CML) is associated with the presence of the Philadelphia chromosome, which is generated by the reciprocal translocation of chromosomes 9 and 22. In the case of L6 (b2a2) mRNA, it is difficult to cleave the abnormal mRNA specifically because the mRNA includes no sequences that can be cleaved efficiently by conventional hammerhead ribozymes near the *BCR-ABL* junction. We recently succeeded in designing a novel maxizyme, which specifically cleaves *BCR-ABL* fusion mRNA, as a result of the formation of a dimeric structure. As an extension of our molecular engineering of maxizymes, as well as to improve their potential utility, we examined whether an analogous conformational change could be induced within a single molecule when two maxizymes were connected via a linker sequence. An active conformation was achieved by binding of the construct to the *BCR-ABL* junction *in trans*, with part of the linker sequence then acting as an antisense modulator *in cis* (within the complex) to adjust the overall structure. Results of studies in vitro in the presence of cetyltrimethylammonium bromide (CTAB) (but not in its absence) suggested that a certain kind of connected maxizyme (cMzB) might be able to undergo a desired conformational change and, indeed, studies in vivo confirmed this prediction. Therefore, we successfully created a fully functional, connected maxizyme and, moreover, we found that the activity and specificity of catalytic RNAs in vivo might be better estimated if their reactions are monitored in vitro in the presence of CTAB.**

Key words: Maxizyme; Ribozyme; Chronic myelogenous leukemia; Allosteric control; CTAB

1. Introduction

The hammerhead ribozyme, which is the smallest natural catalytic RNA identified to date, was first detected in certain small satellite and viroid RNAs [1–5]. Because the catalytic motif is so compact, hammerhead ribozymes have been engineered to act *in trans* to cleave other RNA molecules [6,7]. The *trans*-acting hammerhead ribozyme consists of an antisense section (stems I and III) and a catalytic domain with a flanking stem-loop II section (Fig. 1A) [8]. When stems I and III are designed as antisense sequences complementary to target mRNAs, the hammerhead can be utilized as a potential therapeutic agent or tool for the regulation of expression of a specific gene [8–17].

Chronic myelogenous leukemia (CML), which is a clonal myeloproliferative disorder of hematopoietic stem cells, accounts for about 15–20% of all leukemias [18]. The Philadelphia chromosome (Ph1), which is caused by reciprocal translocation of chromosomes 9 and 22, is characteristically detectable in more than 95% of patients with CML. The breakpoints on chromosome 22 are clustered within a 6-kb region known as the breakpoints cluster region (bcr), while the breakpoints on chromosome 9 are spread over a 90-kb region upstream of exon 2 of c-*abl* [19–24]. The *BCR-ABL* fusion gene, after splicing, generates two different *BCR-ABL* fusion mRNAs, namely K28 b3a2 mRNA and L6 (b2a2) mRNA (Fig. 2). These mRNAs are translated into $p210^{BCR-ABL}$ protein, which has elevated tyrosine kinase activity and causes the malignant cell phenotype [25].

If a ribozyme is to disrupt chimeric *BCR-ABL* mRNA, it must target the junction sequence exclusively. Otherwise, normal mRNAs that share part of the chimeric RNA sequence will also be cleaved by the ribozyme, with resultant damage to host cells. In the case of the K28 *BCR-ABL* chimeric b3a2 RNA sequence (Fig. 2), a potential site of cleavage by a ribozyme, a GUU triplet, is located three nucleotides upstream of the chimeric junction. Thus, a conventionally designed hammerhead ribozyme might be expected to cleave specifically the abnormal mRNA generated from K28 translocations. Indeed, several examples of such cleavage have been reported [26–33]. By contrast, in the case of the b2a2 sequence, which results from L6 translocations, as well as in the case of some K28 translocations, there are no triplet sequences that are potentially cleavable by hammerhead ribozymes within two or three nucleotides of the junction in question. GUC triplets are generally the triplets that are most susceptible to cleavage by a hammerhead ribozyme [34–38], and one such triplet is located 45 nucleotides from the junction (Fig. 2). If this GUC triplet were targeted by a ribozyme, the normal *ABL* mRNA, which shares part of the sequence of the abnormal *BCR-ABL* mRNA, would also be cleaved by the ribozyme, with resultant damage to the host cells [39]. In designing ribozymes that might cleave b2a2 mRNA, we must be careful to avoid cleavage of normal *ABL* mRNA.

We demonstrated previously that shortened forms of (stem II-deleted) hammerhead ribozymes, with low intrinsic activity, form very active dimers with a common stem II (Fig. 1B) [40]. In order to distinguish monomeric forms of conventional minizymes that have low activity from our novel dimers with high-level activity, the latter very active short ribozymes, which are capable of forming dimers, are designated 'maxi-

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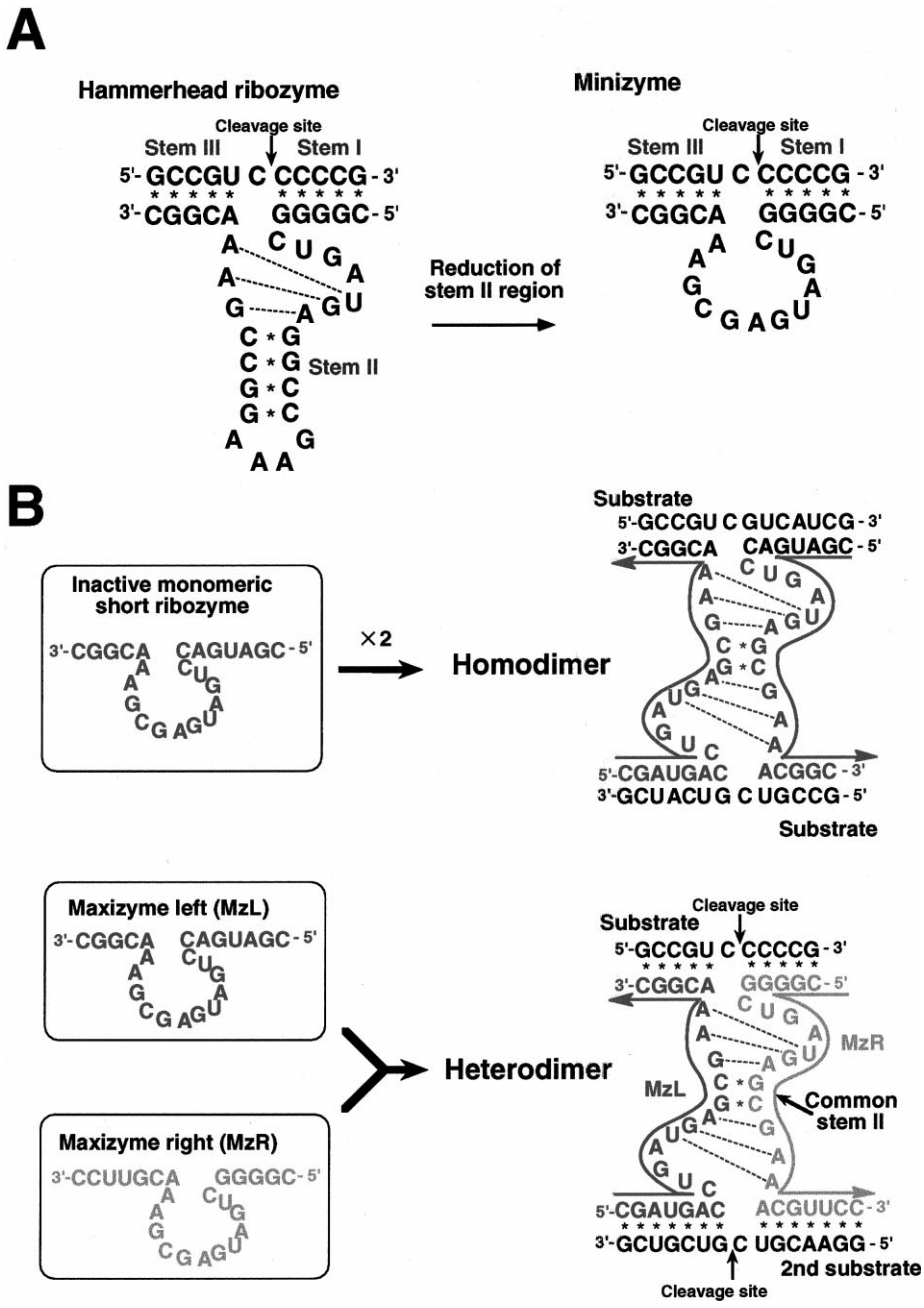


Fig. 1. Secondary structures of a hammerhead ribozyme and of maxizymes. A: The secondary structure of the parental hammerhead ribozyme consists of an antisense section (stems I and III) and a catalytic domain with a flanking stem/loop II section (left). The secondary structure of a monomeric minizyme, namely, a conventional hammerhead ribozyme with a deleted stem/loop II region, is shown on the right. B: Secondary structure of a maxizyme that is capable of forming a homodimer (top) and a heterodimer (bottom). The homodimer has two identical binding sites, while the heterodimer can generate two different binding sites, one of which is complementary to the sequence of one substrate and one of which is complementary to a second substrate. Maxizyme left (MzL) and maxizyme right (MzR) form the heterodimeric maxizyme (bottom).

zymes', abbreviated as 'Mz' [39,41]. The dimers can be homodimeric (with two identical binding sequences) or heterodimeric (with two different binding sequences; Fig. 1B). A heterodimer has two different substrate binding regions and two catalytic domains [41,42]. Because of the two independent catalytic cores, a heterodimer can cleave a single substrate at two independent sites simultaneously (in contrast to homodimeric maxizymes that cleave at one site only) [41]. Moreover, one of the catalytic domains of the heterodimer can be converted to a sensor arm that can allosterically regulate the activity of the maxizyme [39]. Thus, one of our maxizymes was designed to adopt an active conformation only in the presence of an abnormal *BCR-ABL* junction, while the conformation remains inactive in the presence of the normal *ABL* mRNA, as well as in the absence of the abnormal *BCR-ABL* junction (see Fig. 1 of Kuwabara et al. [39]). Studies in vitro and in cultured cells demonstrated that such conformational changes did indeed occur and depended on the presence or absence of the abnormal *BCR-ABL* mRNA [39]. This phenomenon resembles the changes in conformation of allosteric proteinaceous enzymes that occur in response to their effector molecules, and ours was the first demonstration of an artifi-

cially created ribozyme whose catalytic activity is under perfect allosteric control, not only in vitro but also in cultured cells.

In the case of heterodimeric maxizymes in general, dimerization can potentially generate a mixture of inactive (MzR·MzR) dimers (consisting of two identical forms of maxizyme right), inactive (MzL·MzL) dimers (consisting of two identical forms of maxizyme left), and the desired active (MzR·MzL) dimers (consisting of maxizyme right and maxizyme left). Such a mixed population of dimers is one of the reasons for the low activity of some maxizymes, at least in vitro [43]. In order to avoid formation of such inactive homodimers, as well as to enhance the potential utility of maxizymes, we postulated that it might be possible to connect two types of maxizyme (namely, MzR and MzL), transcribed under the control of a single promoter, without sacrificing the activity and specificity of the dimeric maxizyme.

We demonstrate in this paper that the catalytic activity of a specifically designed connected-type maxizyme (cMz) can be controlled allosterically in the presence of the junction in *BCR-ABL* chimeric L6 (b2a2) mRNA. Moreover, although activities of catalytic RNAs in vitro do not necessarily reflect their activities in vivo, we found that the activity and specificity of connected maxizymes in vivo can be better estimated by monitoring their kinetic behaviors in the presence of cetyltrimethylammonium bromide (CTAB) in vitro.

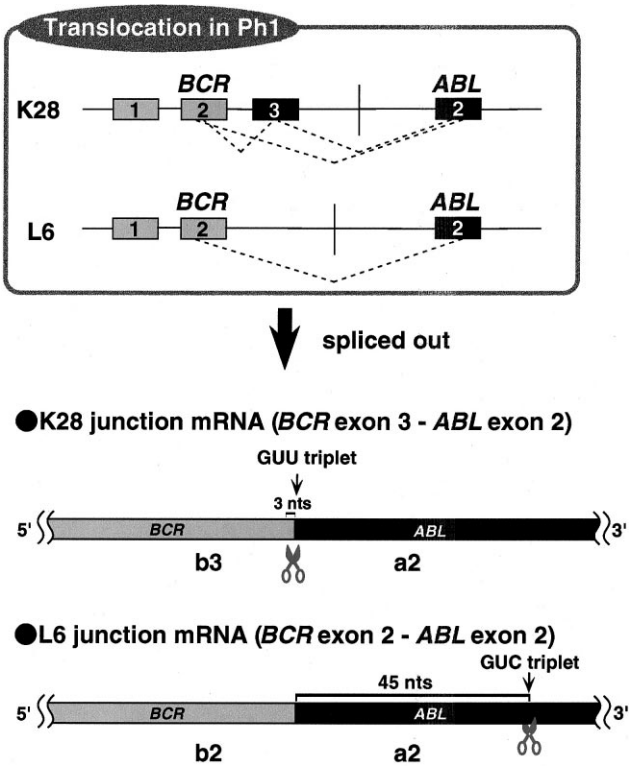


Fig. 2. Translocations and possible sites of cleavage by conventional ribozymes within normal *ABL* mRNA and within abnormal *BCR-ABL* fusion mRNAs. Schematic representations of the two types of chromosomal translocation (K28-type and L6-type, upper panel) that are associated with CML and the corresponding fusion mRNAs are shown (lower section). Boxes in gray represent *BCR* exons and boxes in black represent *ABL* exon 2. Dotted lines connecting *BCR* and *ABL* exons indicate alternative splicing pathways. Ph1, Philadelphia chromosome.

2. Materials and methods

2.1. Transcription of connected maxizymes (cMz)

DNA templates for connected maxizymes (cMz) were synthesized chemically by a DNA/RNA synthesizer (model 394; Perkin Elmer, Applied Biosystems (ABI), Foster City, CA). The templates for T7 transcription had the following sequences: cMz1, 5′-CAC TCA CTG ATG AGA GTT ATT GAT GGT CAG GTT ATA ATA AGA AAG AAA AGA AGG GCT TCT TTC ATC GAA ACC CTG AGG-3′; cMz2, 5′-CAC TCA CTG ATG AGA GTT ATT GAT GGT CAG AAA AAA AAA AAA GAA GGG CTT TCA TCG AAA CCC TGA GG-3′; cMz3, 5′-GAA GGG CTT CTT TCA TCG AAA CCC TGA GGA AAA AAA AAA AAA AAC ACT CAC TGA GAG TTA TTG ATG GTC AG-3′; cMz4, 5′-GAA GGG CTT CTT TCA TCG AAA CCC TGA GGA AAT AAT AAA GAA AGA AAA CAC TCA CTG ATG AGA GTT ATT GAT GGT CAG-3′; cMzA, 5′-GAA GGG CTT CTT TCA TCG AAA CCC TGA GGA ATA GAT CTA AAA GAA TGC TGT GAA AGA AAG CGA ATA CAC TCA CTG ATG AGA GTT ATT GAT GGT CAG-3′; and cMzB, 5′-GAA GGG CTT CTT TCA TCG AAA CCC TGA GGA AAA CTC AAA GTC AAA AAA AAC ACT CAC TAA TGA GAG TTA TTG ATG GTC AG-3′.

Primers were also synthesized for each template, and sense strands included the T7 promoter. Products of the polymerase chain reaction (PCR) were purified on an agarose gel. T7 transcription in vitro and gel-electrophoretic purification of each cMz were performed as described elsewhere [37].

2.2. Synthesis of target substrates

Short substrates for kinetics (S16, *BCR-ABL* pseudo-sub) were chemically synthesized on a DNA/RNA synthesizer. S16 which contained GUC triplet located 45 nucleotides 3′ of the *BCR-ABL* junction had the following sequence: 5′-CCU CAG GGU CUG AGU G-3′. *BCR-ABL* pseudo-sub which contained *BCR-ABL* mRNA sequence around the *BCR-ABL* junction had the following sequence: 5′-CUG ACC AUC AAU AAG GAA GAA GCC CUU C-3′.

DNA templates for L6 *BCR-ABL* substrate RNA and the normal *ABL* substrate RNAs were synthesized chemically. The DNA oligodeoxynucleotide template for L6 *BCR-ABL* substrate RNA consisted of the sequence from 63 nucleotides 5′ of the *BCR-ABL* junction to 58 nucleotides 3′ of the *BCR-ABL* junction. The region of the DNA oligodeoxynucleotide template for the normal *ABL* substrate RNA extended from position 192 to position 283 of normal *ABL* cDNA. Primers were also synthesized for each template, and each sense strand contained the T7 promoter. The products of PCR were gel-purified. T7 transcription in vitro and gel-electrophoretic purification of the *ABL* and *BCR-ABL* mRNA substrates were performed as described elsewhere [37].

2.3. Measurements of kinetic parameters

Measurements of kinetic parameters of reactions catalyzed by cMz1–4 were made with 5′-^{32}P-labeled short substrates (S16). Reaction rates were measured, in 25 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0) and 10 mM NaCl under single-turnover conditions at 37°C with 28-mer *BCR-ABL* (1 μM) as the effector molecule. Reactions were initiated by addition of appropriate amounts of $MgCl_2$ after pre-incubation of a reaction mixture that contained all components except $MgCl_2$ for several minutes at 37°C. Reactions were stopped by removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equivalent volume of a solution that contained 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue. The substrate and the products of the reaction were separated by electrophoresis on a 5% polyacrylamide/7 M urea denaturing gel and were detected by autoradiography. The extent of cleavage was determined by quantitation of radioactivity in the bands of substrate and products with a Bio-Image Analyzer (BAS2000; Fuji Film, Tokyo).

2.4. Specificity and kinetic analysis of the cleavage of BCR-ABL chimeric L6 (b2a2) mRNA by cMzA and cMzB in the presence of CTAB

For the examination of specificity, when reactions were catalyzed by cMzA and cMzB, 5′-^{32}P-labeled long substrates (L6 *BCR-ABL* substrate RNA and normal *ABL* substrate RNA) were used. The substrates were 5′-labeled with [^{32}P]ATP by T4 polynucleotide kinase

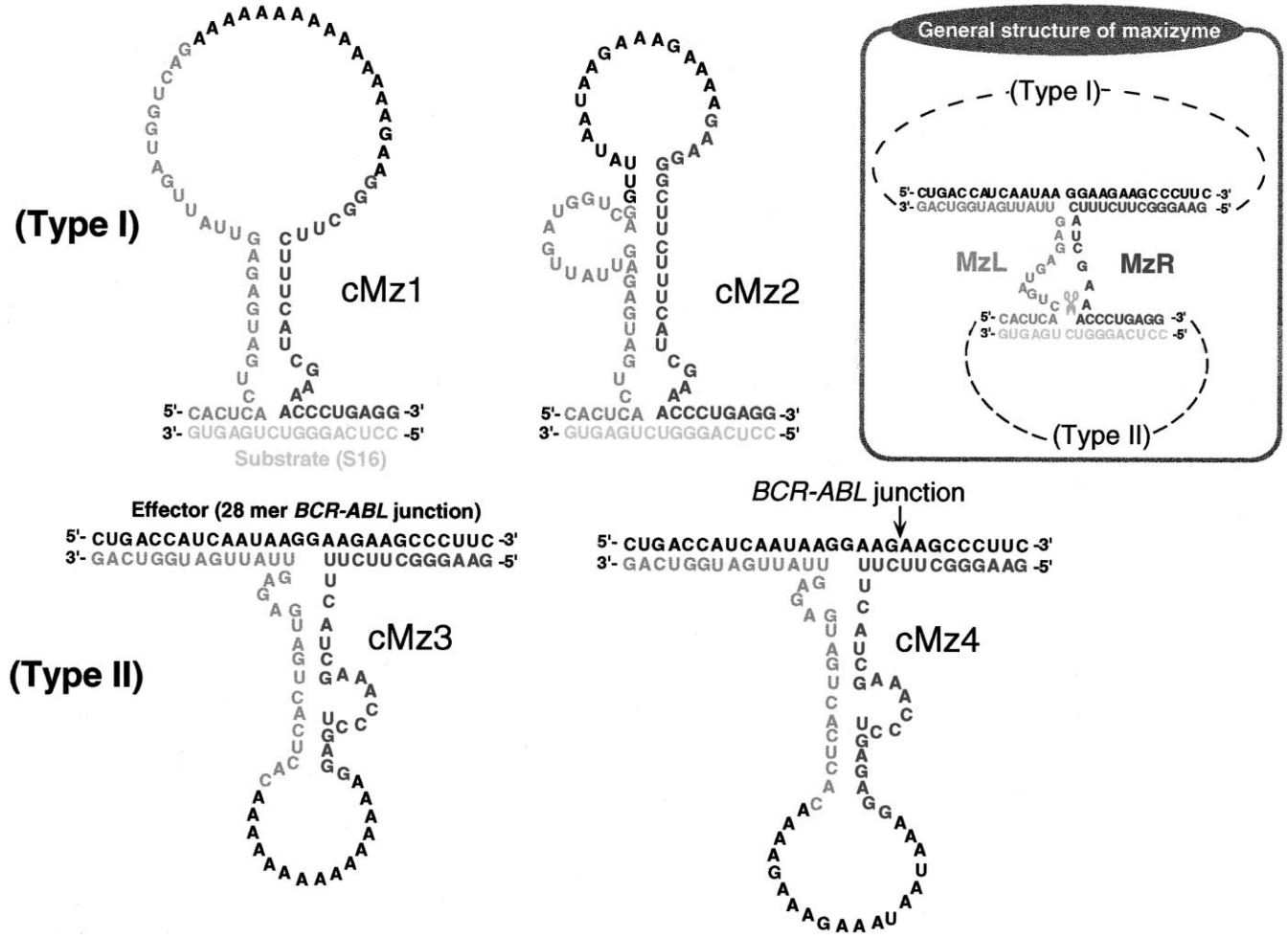


Fig. 3. Secondary structures of a connected maxizyme (cMz) and its target. Connected maxizymes are generated in two ways, as indicated by type I and type II. CMz1 and cMz2 belong to type I construct. CMz3 and cMz4 belong to type II construct.

(Takara Shuzo, Kyoto). In order to characterize in further detail the properties of cMz4, cMzA and cMzB, we determined the kinetic parameters of cleavage using long substrates (see the table at the bottom of Fig. 6). Reactions were performed, in 25 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0) and 10 mM NaCl under single-turnover conditions at 37°C, in the presence or absence of 50 μM CTAB (Sigma, St. Louis, MO). Reactions were initiated by the addition of $MgCl_2$ to a buffered solution that contained each enzyme with the substrate, and each resultant mixture was then incubated at 37°C. Reactions were stopped by removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equal volume of a solution that contained 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue. Finally, reaction mixtures were subjected to electrophoresis on 5% polyacrylamide/7 M urea gel. Rate constants are averages from three sets of experiments.

Table 1
Kinetic parameters for reactions catalyzed by the first generation of connected maxizymes[a]

Ribozyme	k_{cat} (min^{-1})	K_d(app) (μM)
cMz1	0.015	0.06
cMz2	0.016	0.05
cMz3	0.002	0.014
cMz4	0.003	0.024

[a]Kinetic measurements were made in 50 mM Tris-HCl (pH 8.0) and 25 mM $MgCl_2$, under enzyme-saturating conditions at 37°C with the 28-mer *BCR-ABL* junction-containing RNA (1 μM) as the effector molecule. Rate constants are averages from three sets of experiments.

2.5. Construction of plasmids for the expression of connected maxizymes

Chemically synthesized oligonucleotides encoding each ribozyme (cMzA, cMzB) and the polymerase (pol) III termination sequence [44] were converted to double-stranded sequences by PCR. After digestion with *Csp*45I and *Sal*I, each appropriate fragment was cloned downstream of the promoter of the gene for $tRNA^{Val}$ pV (in which the chemically synthesized promoter of a human gene for $tRNA^{Val}$ had been ligated between the *Eco*RI and *Sal*I sites of the pMX puro vector) [45]. The sequences of the constructs were confirmed by direct sequencing.

2.6. Assays of reporter activity after transient transfection

The vectors shown in Fig. 7A were used to transfect HeLa cells in combination with Lipofectin Reagent (Gibco-BRL, Rockville, MD). Luciferase activity was measured with a Pica Gene kit (Toyo-inki, Tokyo) as described elsewhere [46]. In order to normalize the efficiency of transfection by reference to β-galactosidase activity, cells were co-transfected with the pSV-β-galactosidase control vector (Promega, Madison, WI) and then the chemiluminescent signal due to β-galactosidase activity was quantitated with a luminescent β-galactosidase genetic reporter system (Clontech, Palo Alto, CA) as described previously [46]. The results shown are the averages of results from more than three sets of experiments.

3. Results and discussion

3.1. Design of connected maxizymes (cMz1–cMz4) and their specificity with respect to the cleavage of BCR-ABL chimeric L6 (b2a2) mRNA

For the potential future application of dimeric maxizymes as a potential treatment for CML, we attempted to design new connected maxizyme motifs that would specifically cleave *BCR-ABL* chimeric L6 (b2a2) mRNA. First, we designed and synthesized, by T7 transcription from the corresponding DNA templates, four connected maxizymes, cMz1–4 (Fig. 3). Maxizymes can be connected in at least two ways, as indicated by type I and type II in Fig. 3. Two constructs, cMz1 and cMz2, belong to the type I construct in that the sensor arms of MzR and MzL, which recognize the abnormal *BCR-ABL* junction, were connected by a linker sequence between the 5′-end of MzR and the 3′-end of MzL (dotted line in the general structure of maxizyme; right panel). In the type II constructs, cMz3 and cMz4, MzR and MzL were connected at the substrate recognition arms, which correspond to stems I and III of the parental hammerhead ribozyme (Fig. 1A, left), by a linker that connected the 5′-end of MzL and the 3′-end of MzR.

In order to examine the activity of the maxizymes in vitro, we at first used a short 16 nucleotides *BCR-ABL* substrate that corresponded to the target (cleavage) site (the substrate used, S16, is indicated in Fig. 3). Kinetic measurements of the k_{cat} and K_d(app) values listed in Table 1 were made in 25 mM

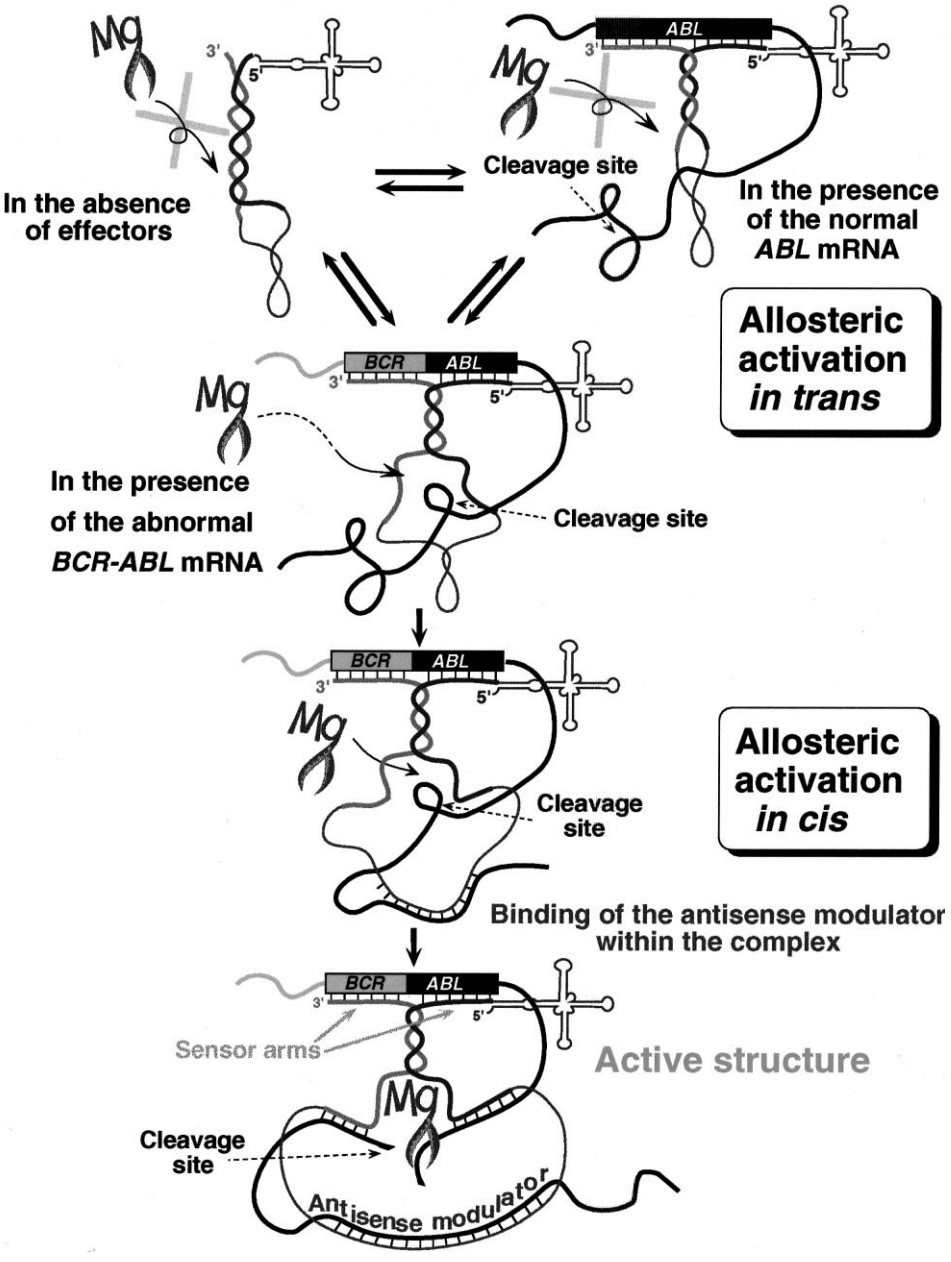


Fig. 4. Schematic representation of allosteric control with two types of activation by a novel connected maxizyme. A conformational change should be induced within a single molecule when maxizymes are connected via a linker sequence. The active conformation is achieved by binding to the *BCR-ABL* junction *in trans*. Then a part of the linker sequence acts as an antisense modulator *in cis* to adjust the overall structure. The alternative sequence of events is also possible.

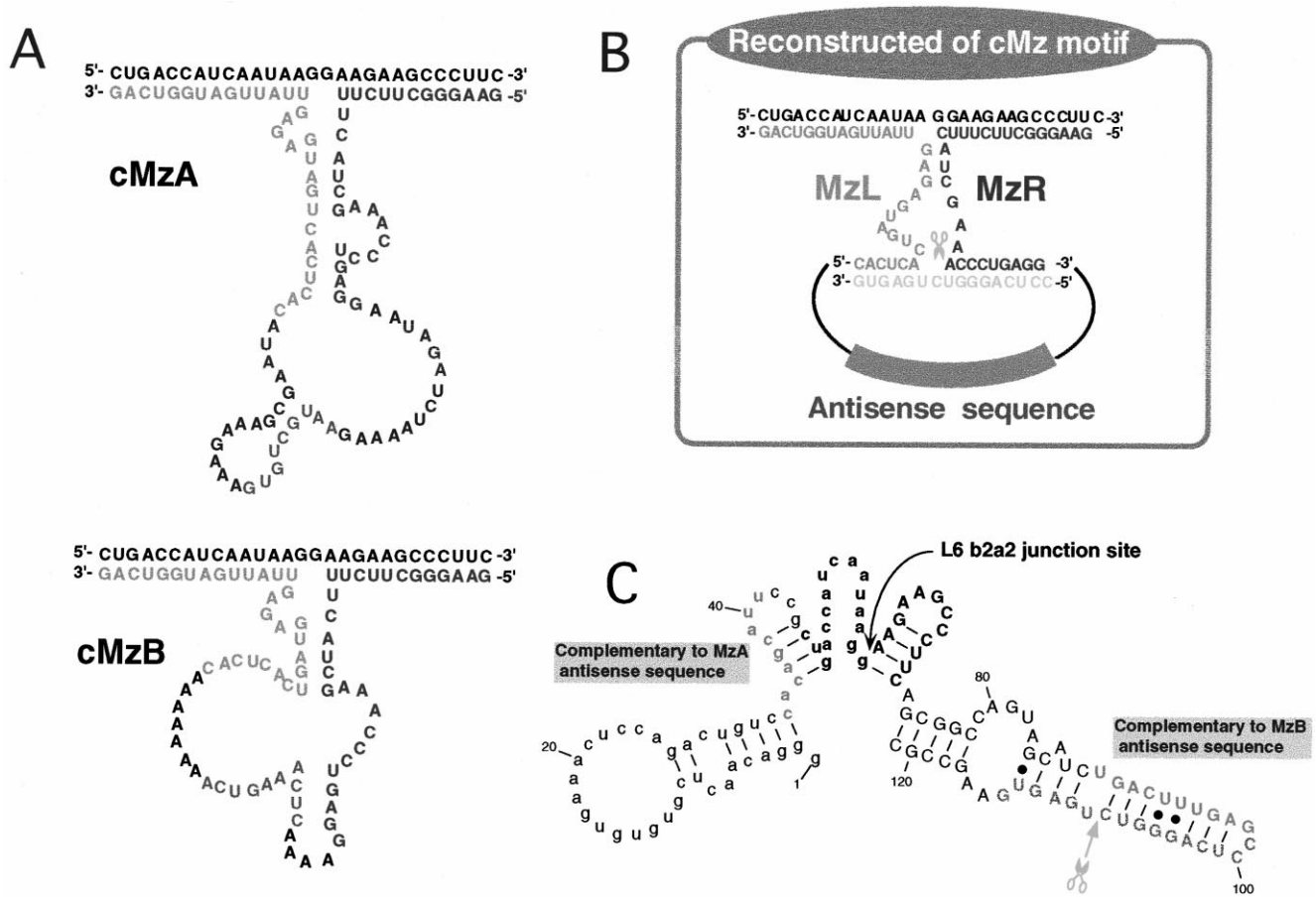


Fig. 5. Secondary structures of the connected maxizyme (cMz) and the target. A, B: Secondary structures of two connected maxizymes, cMzA and cMzB, with antisense modulator sequences. To improve the type II motif, an antisense modulator was introduced within the linker sequence so that the size of the population of correctly folded complexes would be increased by formation of basepairs with part of the target mRNA, with resultant correct basepairing of the maxizyme with the cleavage site. C: The secondary structure of *BCR-ABL* mRNA around the b2a2 junction. The *BCR* exon 2 sequence is depicted by lower-case letters and that of the *ABL* exon 2 sequence is depicted by capital letters. Complementary sequences to each antisense modulator of cMzA and cMzB are also shown. Cleavage site by connected maxizymes is indicated with scissors.

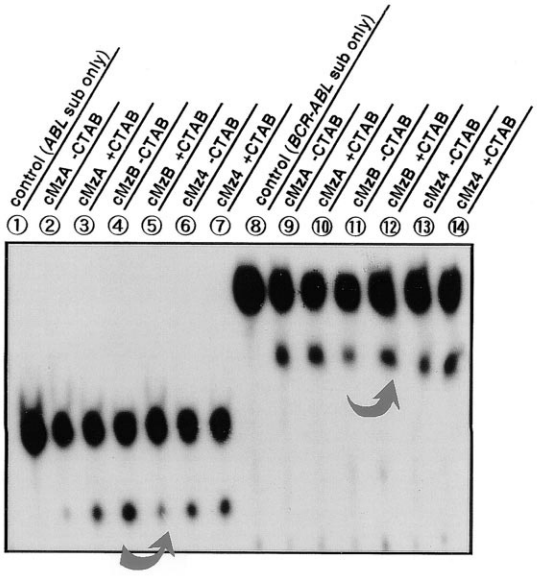
$MgCl_2$ and 50 mM Tris-HCl (pH 8.0), under enzyme-saturating (single-turnover) conditions at 37°C with the 28-mer *BCR-ABL* junction-containing RNA (1 μM) as the effector molecule, and the cleavage products were analyzed on a denaturing polyacrylamide gel as described in Section 2. Comparison of kinetic parameters revealed that type I connected maxizymes were as active as their parental dimeric maxizyme in vitro, and they were more active than type II maxizymes. However, unfortunately, those type I maxizymes (cMz1 and cMz2) with higher activity cleaved the substrate (S16) not only in the presence of the 28-mer effector molecule (*BCR-ABL* junction) but cleavage also occurred at a similar rate in the absence of the effector molecule, whereas type II maxizymes (cMz3 and cMz4) with lower activity retained some specificity (data not shown). Therefore, all (for cMz1 and cMz2) or a significant part (for cMz3 and cMz4) of the specificity was lost by simply connecting two independent maxizyme units (MzR and MzL).

Since specificity is one of the most important factors in the design of maxizymes that are to disrupt a chimeric mRNA and since cleavage of normal mRNA must be avoided to avoid damage to host cells, we decided that the molecular design of connected maxizymes would have to be based on the type II construct.

3.2. *Second generation of connected maxizymes (cMzA and cMzB) and re-examination of their specificity with respect to the cleavage of BCR-ABL chimeric L6 (b2a2) mRNA in the presence and in the absence of CTAB*

Since the first generation of simply connected maxizymes (cMz) failed to exhibit reasonable specificity in vitro, we decided to introduce additional features that would favor the formation of a correctly folded complex. To improve the type II motif, we attempted to increase the specificity with respect to the cleavage of *BCR-ABL* chimeric L6 (b2a2) mRNA by introducing an antisense modulator within the linker sequence. We hoped that the size of the population of correctly folded complexes would increase as a result of the formation of basepairs with part of the target mRNA, as shown schematically in the lower part in Fig. 4. The inserted antisense modulator sequences in the two connected maxizymes (cMzA and cMzB) are indicated in Fig. 5.

In order to examine the activity and specificity of T7 transcribed cMzA and cMzB in vitro, we prepared by T7 transcription the normal *ABL* substrate [47] and the chimeric *BCR-ABL* substrate (Fig. 5C), respectively, of 92 and 121 nucleotides, that are expected to be better substrates than shorter ones in the prediction of the connected maxizymes'



	-CTAB		+CTAB	
	k_{obs} (min^{-1}) X 10^3			
	ABL (92mer)	*BCR-ABL* (121mer)	*ABL* (92mer)	*BCR-ABL* (121mer)
Enzymes				
cMzA	0.58	1.00	1.29	1.41
cMzB	1.74	0.73	0.44	1.25
cMz4	1.09	0.82	1.16	1.45

Fig. 6. The specificity of cMz with respect to the cleavage of *BCR-ABL* substrate in the presence and in the absence of CTAB. Kinetic parameters for the cleavage of *BCR-ABL* mRNA (121-mer substrate) and *ABL* mRNA (92-mer), in the presence and in the absence of CTAB, are also shown in the table at the bottom. All reactions were measured in 50 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$ and 10 mM NaCl in the presence or absence of 50 μM CTAB under enzymes saturating (single-turnover) conditions at 37°C. Rate constants are averages from three sets of experiments.

specificity in vivo. Moreover, the cationic detergent CTAB, which is known to enhance strand displacement of nucleic acids, was also used in the prediction in vitro of the connected maxizymes' specificity in vivo, since our previous study demonstrated that the activity of ribozymes in vivo can be better estimated by monitoring ribozyme kinetics in the presence of CTAB in vitro [43]. We performed kinetic analyses by running reactions in mixtures that contained 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 25 mM $MgCl_2$, at 37°C under single-turnover conditions, using a fixed concentration of the connected maxizyme, cMzA or cMzB (50 nM), and with or without a fixed concentration of CTAB (50 μM) that was slightly above its critical micelle concentration (CMC).

To our surprise, when the longer substrates were used, cMz4, which showed some specificity with the short 16-mer substrate, cleaved both the abnormal *BCR-ABL* substrate (lane 13 in Fig. 6) as well as the normal *ABL* substrate (lane 6) with a similar efficiency in the absence of CTAB (see rate constants at the bottom of Fig. 6). Upon addition of CTAB, cMz4 gained limited specificity (see lanes 7 and 14, and the table in Fig. 6). In the case of cMzA, CTAB accelerated the cleavage of not only the abnormal *BCR-ABL* substrate (lanes 9 and 10) but also, and even to a greater extent, the normal *ABL* substrate (compare lanes 2 and 3, and see the table for rate constants).

Fortunately, cMzB, which did not show specificity in the absence of CTAB (lanes 4 and 11), started to demonstrate specificity upon addition of CTAB (lanes 5 and 12, and rate constants in Fig. 6). In designing the second generation of connected maxizymes, cMzA and cMzB, we took care to ensure that the linker region would not form intramolecular basepairs within cMz. However, it was impossible to predict, at least by computer folding analysis, which cMz would have greater specificity.

Many so-called facilitators have recently been identified that significantly increase the rates of formation of RNA duplexes and of ribozyme-catalyzed reactions [48–59]. These facilitators include nuclear proteins, the HIV-1 nucleocapsid protein, and cationic detergents. It seems possible, therefore, that the capacity of ribozymes for the rapid and specific cleavage of RNAs in vivo might be enhanced by such facilitators if ribozymes or substrates in an inactive conformation could be converted to active forms in vivo. Our previous study demonstrated that various kinds of facilitator in vivo that function similarly to CTAB might enhance the activity of dimeric maxizymes and that CTAB appears to be useful in predicting activities of ribozymes in vivo [43]. As will be shown below, the present study demonstrates that CTAB appears to be useful in predicting not only the relative activity of ribozymes but also the specificity of the maxizymes, namely, as predicted in this section in the presence of CTAB, only cMzB demonstrates high specificity in cultured cells.

3.3. *Connected maxizymes under the control of a human $tRNA^{Val}$ promoter and their activities in vivo*

For application of connected maxizymes to gene therapy, the effective delivery into cells and the high-level expression of connected maxizymes are obviously critically important. In our laboratory, we have exploited an endogenous delivery system that is quite efficient [60]. We use the promoter of a human gene for $tRNA^{Val}$ [13,15,61–63] that is recognized by RNA pol III [36,44]. While pol II promoters might allow tissue-specific or regulatable expression, we anticipated that pol III transcripts might be expressed at significantly higher levels. It would clearly be advantageous to use a strong promoter for transcription of ribozymes and maxizymes because high-level expression would be more likely to ensure significant suppression of the expression of a specific gene [60]. The $tRNA^{Val}$-expression system resulted in high-level activity of ribozymes and maxizymes in cultured cells [13,15,39,41,60]. Therefore, we embedded cMzA and cMzB similarly in the 3′ portion of the human gene for $tRNA^{Val}$ (Fig. 7). To examine the activity and the specificity of $tRNA^{Val}$-driven maxizymes, we used a reporter construct in a transient assay (Fig. 7A). We co-transfected HeLa cells with various expression plasmids that encoded appropriate maxizymes under the control of the human $tRNA^{Val}$-promoter, as well as a target gene-expressing plasmid that encoded a chimeric target *BCR-ABL* sequence (or *ABL* alone) and a gene for luciferase, pB2A2-luc (or p*ABL*-luc), as described in detail in [39]. The luciferase activity recorded when we used the target gene-expressing plasmid and of the $tRNA^{Val}$-portion-expressing plasmid (pV) was taken as 100% (Fig. 7B). In accord with the results of kinetic measurements in the presence of CTAB, one of the

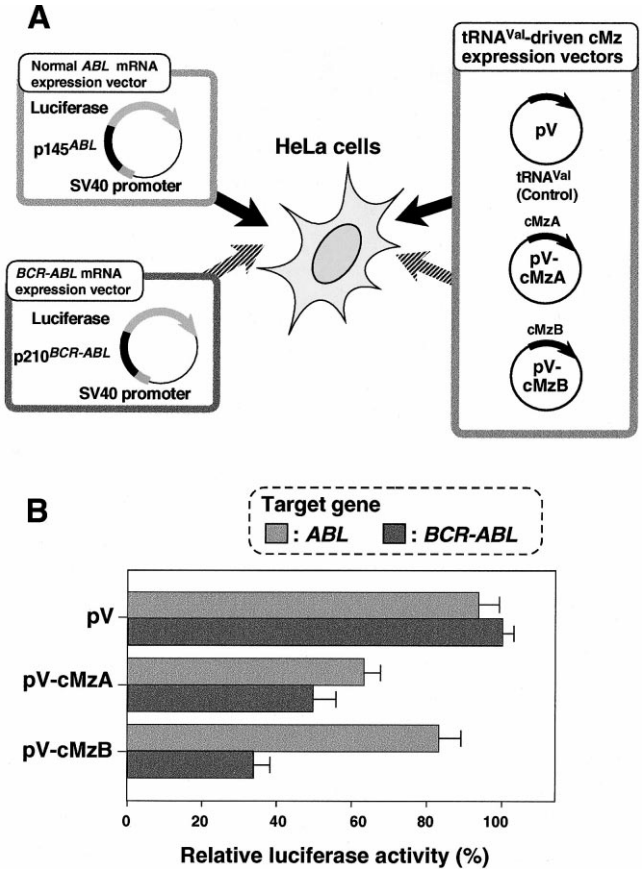


Fig. 7. Intracellular activities and specificities of $tRNA^{Val}$ enzymes in HeLa cells. A: Assay system for measurements of activities of $tRNA^{Val}$ enzymes in HeLa cells. B: The effects of $tRNA^{Val}$ enzymes on the chimeric *BCR-ABL*-luciferase and *ABL*-luciferase genes. Luciferase activity was normalized by reference to the efficiency of transfection which was determined by monitoring activity of a co-transfected gene for β-galactosidase (see Section 2).

connected maxizymes (cMzB) was significantly more specific, in cleaving the abnormal *BCR-ABL*-luciferase gene without damaging the normal *ABL*-luciferase gene, than the other connected maxizymes (cMzA).

In conclusion, for the future treatment of CML by ribozymes, we designed and analyzed new RNA motifs, putting some emphasis on the specificity with respect to the cleavage of *BCR-ABL* chimeric L6 (b2a2) mRNA. We confirmed that cMzB retained the specificity with respect to the cleavage of *BCR-ABL* chimeric L6 (b2a2) mRNA. In the case of cMzA, the antisense modulator complementary to part of the *BCR* was included in the linker sequence. On the other hand, in the case of cMzB, the antisense modulator complementary to part of the *ABL* was included in the linker sequence. Although it was not possible to predict their activity and specificity from their computer-predicted structures nor from their kinetic behaviors in the absence of CTAB, it is significant that not only their activity but also their specificity in vivo could be estimated from their kinetics in vitro when reactions were carried out in the presence of CTAB. These findings suggest that, even though the activities and specificities in vitro of ribozymes in general do not necessarily reflect their activities in vivo, it might be possible to predict their activities and specificities in vivo by monitoring their activities and specificities in vitro in the presence of CTAB.

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