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Review

MAXIZYMEs: Allosterically controllable ribozymes with biosensor functions

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Ribozymes are catalytic RNAs that can cleave RNAs at specific sites, thus they have been employed to degrade a target mRNA *in vivo*. Development of allosterically controllable ribozymes is of great current interest, but it remained difficult to furnish such functions to ribozymes in cultured cells or in animals. Recently, we designed allosterically controllable ribozymes termed maxizymes, which have sensor arms that recognize target mRNA sequences and, in the presence of such target sequences only, they form a cavity that can capture catalytically indispensable Mg^{2+} ions, cleaving the target. The maxizyme was applied to therapy for chronic myelogenous leukemia (CML). It cleaved specifically the chimeric *BCR-ABL* mRNA, which caused CML, without damaging the normal *ABL* or *BCR* mRNA in mammalian cells and also in mice, providing the first successful example for allosteric control of the activity of artificial ribozymes *in vivo*.

Keywords: Ribozyme, Allosteric, Molecular switch, Biosensor, Hammerhead ribozyme, Maxizyme.

Ribozymes Catalytic RNAs, related to cleavage or ligation, are called ribozymes. Hammerhead, hairpin, hepatitis delta virus (HDV) and Newlospolar VS ribozymes; group I and II introns; the RNA subunit of RNase P; and ribosomal RNA (Alberts et al., 1994; Birikh *et al.*, 1997; Zhou and Taira, 1998; Doudna, 1998; Walter and Burke, 1998; Carola and Eckstein, 1999; Gesteland *et al.*, 1999; Lilley, 1999; Scott, 1999; Warashina *et al.*, 1999a; 2000; Kim and Park, 2000) are representative ribozymes that exist in nature. Recently, a larger number of artificial ribozymes with diverse range of catalytic activities have been created by using *in vitro* selection procedure (Famulok, 1999; Roberts and Ja, 1999; Li and Breaker, 1999). The rapidly developing field of RNA catalysis is of particular current interest not only because of the intrinsic catalytic properties of ribozymes but also because of their potential utility as therapeutic agents and specific regulators of gene expression. To date, numerous studies directed towards the application of ribozymes in vivo have been performed and many successful experiments, which aimed at applying ribozymes for the suppression of specific gene expression in different organisms, have been reported (Birikh *et al.*, 1997; Yu *et al.*, 1995; Bertrand *et al.*, 1997; Kawasaki *et al.*, 1998; Lan *et al.*, 1998; Plehn-Dujowich and Altman, 1998; Kuwabara *et al.*, 1998a; Kuwabara *et al.*, 1998b; Kuwabara *et al.*, 1999; Koseki *et al.*, 1999; Warashina *et al.*, 1999b; Tanabe *et al.*, 2000a).

A hammerhead ribozyme, which is generally used as a molecular scissors, is illustrated in Fig. 1. The structure of the catalytic core of a hammerhead ribozyme is stabilized by stem regions and stems I and III are used as substrate recognition sites (Birikh *et al.*, 1997; Zhou and Taira, 1998; Carola and Eckstein, 1999; Warashina *et al.*, 1999a). The RNA cleavage reaction by ribozymes requires the presence of a divalent metal ion such as magnesium (Birikh *et al.*, 1997; Zhou and Taira, 1998). Therefore, hammerhead ribozymes are recognized to be metalloenzymes. The catalytic domain of the hammerhead ribozyme is necessary for capturing catalytically indispensable Mg^{2+} ions, but various changes are possible in other regions of the molecule.

Allosterically controlled ribozymes Most mechanisms of

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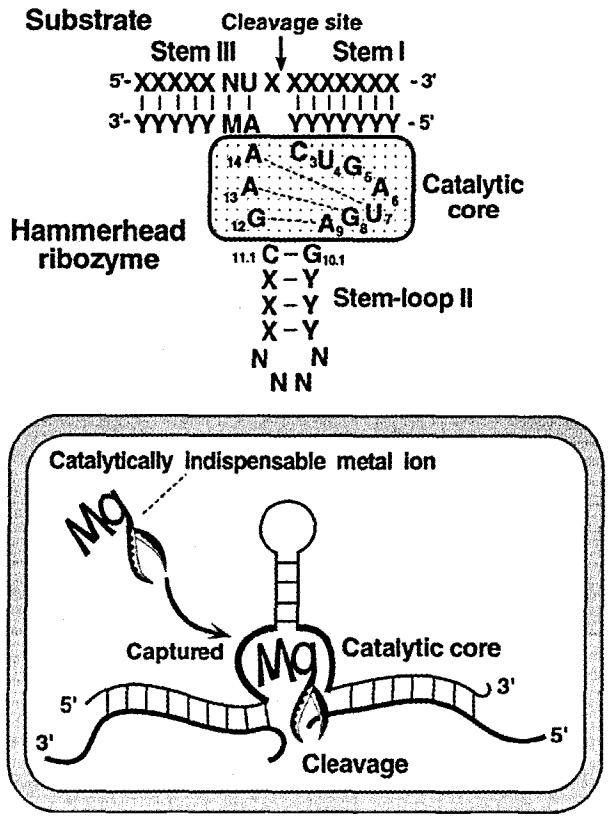

Fig. 1. Secondary structure of the hammerhead ribozyme. The hammerhead ribozyme consists of the substrate-binding region (stems I and III) and a catalytic core (Pink) with a stem-loop II region. By using a construct where the substrate-binding region of the ribozyme is complementary to the target RNA (location marked by "X-Y" and "N-M" in the substrate-binding region), one can create molecular scissors to cleave the RNA in a site-specific manner. When the catalytic core captures the catalytically indispensable Mg^{2+} ions, the cleavage occurs only after the site consisting of the sequence NUX (N, any base; X, A, C, and U).

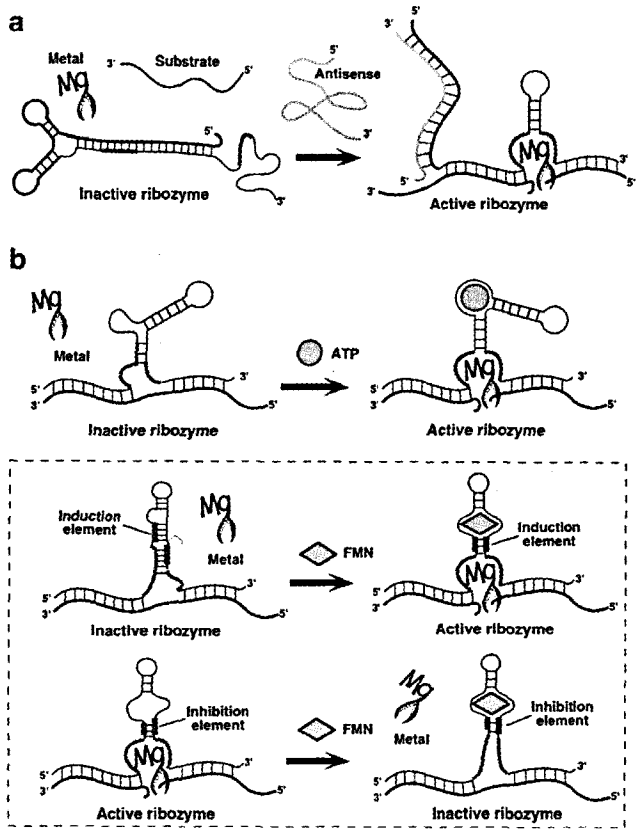

Fig. 2. Rational designs of allosteric ribozymes. (a) Allosteric ribozyme under the regulation of antisense molecule. The ribozyme carrying an effector-recognition domain stably forms an inactive catalytic core structure in the absence of the effector antisense. (b) Allosteric ribozymes under the control of small chemical compounds (Aptazyme). ATP-, theophylline- and flavin mononucleotide- (FMN-) specific aptamers were used as the regulatory domain for the activity of the hammerhead ribozymes. Ligand-specific molecular switches (induction and inhibition) were created by coupling preexisting catalytic and receptor domains of aptamers via communication module.

cell function are controlled in complicated manners by various protein enzymes. Allosteric control of the enzyme activity by effector molecules such as nucleotides, phosphate, and coenzymes is an essential for the delicate regulation of cell function such as metabolisms and signal transductions (Alverts *et al.*, 1994). An effector molecule binds to the site distant from the active site of the enzyme, causing the conformational change in the structure of the enzyme, resulting in regulating the enzymatic activity (Gesteland *et al.*, 1999). For example, in the case of the activation of an enzyme via a phosphorylation, two negative charges of a bound phosphate induce a conformational change by interacting with positively charged regions (side chains of amino acids such as a lysine, an arginine and a histidine) of the proteinaceous enzyme.

The cleavage activity of ribozymes is achieved not by the specific sequence, but rather by the tertiary folding of RNA strands and the RNA conformation which can possess catalytic activities (Gesteland *et al.*, 1999; Birikh *et al.*, 1997; Zhou and Taira, 1998; Doudna, 1998; Walter and Burke, 1998; Carola and Eckstein, 1999; Lilley, 1999; Scott, 1999; Warashina *et al.*, 1999a; Warashina *et al.*, 2000). The hammerhead ribozyme is a representative ribozyme that has been extensively studied for the development of the allosteric ribozymes focused here (Birikh *et al.*, 1997; Zhou and Taira, 1998; Doudna, 1998; Carola and Eckstein, 1999; Warashina *et al.*, 1999a; Warashina *et al.*, 2000; Marshall and Ellington, 1999; Soukup and Breaker, 1999a). Slight change in the conformation of the catalytic core of a hammerhead ribozyme can easily prevent the metal ions from participating in the chemical cleavage or can prevent the metal ions from binding to this site, with a resultant dramatic loss of the activity. Therefore, some inhibitor molecules that bind to the site apart from the active site such as the stem-loop II region induced

the conformational change in the catalytic core of the ribozyme, suppressing allosteric cleavage reation of the hammerhead ribozyme. Porta and Lizardi (Porta and Lizardi, 1995) reported that the addition of an antisense RNA and/or DNA with a sequence complementary to the stem-loop II region allosterically inhibits the activity of a hammerhead ribozyme. As illustrated in Fig. 2a, they engineered a hammerhead ribozyme carrying an additional effector-recognition domain, which stably forms an inactive structure in the absence of the effector molecule. The formation of the active state, with a cavity for the metal ion-binding, of the ribozyme (Fig. 2a, right) is triggered by a specific interaction with a DNA (or RNA) effector molecule that is complementary to the effector-recognition site.

At first, the creation of allosteric ribozymes was achieved by replacing the stem-loop II region of the hammerhead ribozymes simply with an aptamer, together with appropriate linker-stems (Tang and Breaker, 1997; Araki *et al.*, 1998; Tang and Breaker, 1998; Soukup and Breaker, 1999b). ATP-, theophylline- and flavin mononucleotide- (FMN-) specific aptamers were successfully used as the regulatory domain of the activity of the hammerhead ribozymes (Fig. 2b). Breaker and his co-workers systematically investigated about the linker-stems between the ribozyme and the aptamer and they found that, only by changing the sequence and/or length of this element, the ribozymes with the same aptamer can be regulatable not only through allosteric activation but also through allosteric inhibition (Fig. 2b) (Tang and Breaker, 1997; Soukup and Breaker, 1999b). Moreover, they succeeded in the isolation of two kinds of universal linker elements (communication module) by using in vitro selection procedure (Soukup and Breaker, 1999c). One is an induction element that is proposed to stabilize the active state of each allosteric ribozymes with ATP-, theophylline- or FMN-specific aptamer upon binding to this module of the corresponding small molecule (Fig. 2b), and another is an inhibition element that is proposed to stabilize the inactive state, instead (Fig. 2b). The concept of the universal linker indicates that any kind of aptamers is available as a regulatory module for allosteric ribozyme by attaching the aptamer through this linker and, more importantly, that ribozymes can be available as detecting reagents of a variety of small molecules in diagnostic assays (Marshall and Ellington, 1999; Soukup and Breaker, 1999a; Robertson and Ellington, 1999; Soukup and Breaker, 1999c). It suggests that allosteric ribozymes are useful as molecular switches and also as biosensors. Indeed, an artificial ribozyme ligase was also found to be regulatable by ATP, theophylline and FMN as well (They named these allosteric ribozymes as aptazymes with aptamer-based regulatory domain) (Robertson and Ellington, 1999; 2000).

Maxizymes, allosterically controllable ribozymes Allosteric ribozymes discussed below are developed as a result of studies to shorten the hammerhead ribozyme. We succeeded to create an allosteric ribozyme, the maxizyme, which works as a dimer with significant specificity and activity both *in vitro* and *in vivo* (Kuwabara *et al.*, 1998a; 1998b; 1999; Tanabe *et al.*, 2000a; 2000b). This dimeric allosteric ribozyme demonstrated successfully for the first time that an artificially created enzyme could be useful not only *in vitro* but also *in vivo* as a gene-inactivating agent and also as a biosensor (see below). As indicated below, we have developed novel RNA dimer motifs as a result of studies on "minizymes" to shorten the ribozymes. The term "minizymes" was the name given to the shortened (minimized) ribozymes, but it also had a negative connotation as a ribozyme with extremely low activity (minimum). However, the novel ribozymes designed by us have extremely high activity in the cell. Thus, we have named these new highly active minimized dimeric ribozymes "*maxizyme*" [*m*inimized, *a*ctive, *x*-shaped (functions as a dimer), and *i*ntelligent (allosterically controllable) ribo*zyme*].

Shortened hammerhead ribozymes which function as dimers The RNA cleavage by ribozymes in general requires the presence of a divalent metal ion such as magnesium (Gesteland *et al.*, 1999; Birikh *et al.*, 1997; Zhou and Taira, 1998; Doudna, 1998; Walter and Burke, 1998; Carola and Eckstein, 1999; Lilley, 1999; Scott, 1999; Warashina *et al.*, 1999a; Warashina *et al.*, 2000; Famulok, 1999). The catalytic domain of hammerhead ribozymes captures the catalytically indispensable Mg^{2+} ions. Controlling this capturing function of the metal via a conformational change in the catalytic core of ribozymes is the universal key among creations of various allosteric ribozymes. Frequently, the conformational change in the catalytic core region was designed by substituting the stem-loop II region with specific RNA motif as described above. On the other hand, to create a smaller version of the hammerhead ribozyme called as a minizyme, the stem-loop II region has also been substituted with a short linker (Fig. 3a) (McCall *et al.*, 1992; Tuschl and Eckstein, 1993; Fu *et al.*, 1994; Long and Uhlenbeck, 1994). Unfortunately, such minizymes were found to be quite low in the activities. However, we found that a minizyme completely lacking the stem-loop II region had activity essentially equivalent to the wild-type (Amontov and Taira, 1996). Kinetic and NMR analyses indicated that the shortened ribozyme was essentially inactive as a monomer, but exhibited high catalytic activity as a dimer (Fig. 3b) (Amontov and Taira, 1996; Kuwabara *et al.*, 1998a).

We initially called this ribozyme a "dimeric minizyme", but latter renamed this molecule "maxizyme" (Kuwabara *et al.*, 1998a; 1998b; 1999; Tanabe *et al.*, 2000a; 2000b). In addition, we designed a heterodimeric system made of two different monomers, maxizyme left (MzL) and maxizyme right (MzR) (Kuwabara *et al.*, 1996; 1998b; 1999). In this system, only when MzL and MzR form a dimer, the substrate is cleaved. It is noteworthy that maxizymes have two substrate-binding regions. We have taken this feature to develop an allosteric version with sensor function.

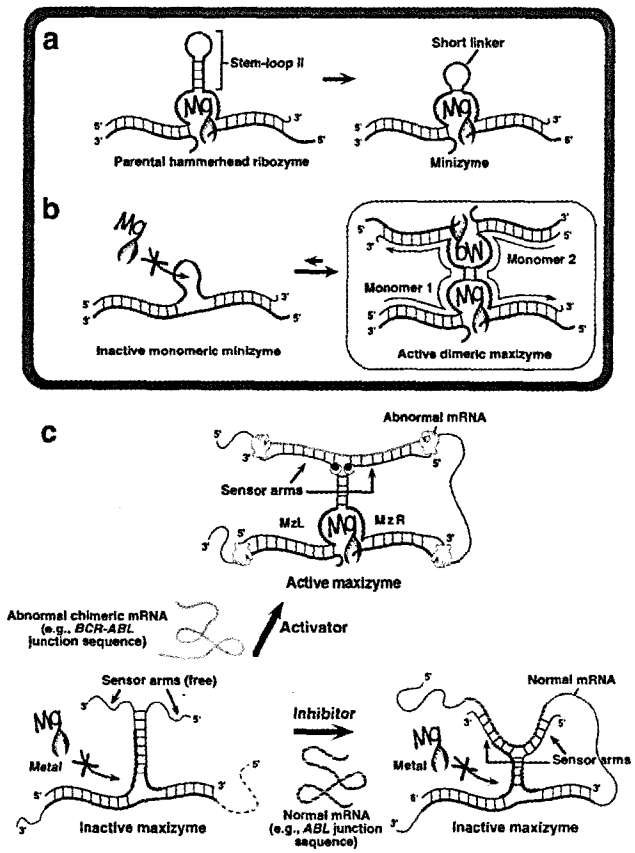

Fig. 3. Development of an allosterically controllable maxizyme. (a) Secondary structures of parental hammerhead ribozyme and a conventional minizyme. (b) Formation of active (homo)dimeric maxizyme. A monomeric minizyme, namely, a hammerhead ribozyme with a deleted stem-loop II region, is inactive. (c) Formation of an active or inactive maxizymes via dimerizations regulated allosterically 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], the other is complementary to a cleavable sequence. In order to achieve high substrate-specificity, the maxizyme should be in an active conformation only in the presence of the abnormal *BCR-ABL* junction (upper panel), while the conformation should remain inactive in the presence of normal *ABL* mRNA (lower right panel) or in the absence of the *BCR-ABL* junction (lower left panel).

Design of an allosterically controllable maxizyme Before describing about the construction process of our allosteric version, we state the background for the development of maxizymes. Maxizymes were designed to overcome the problem in which conventional ribozymes had fallen, rather than to create allosteric ribozymes from the beginning. Although conventional hammerhead ribozymes can target essentially any RNA at specific sites, there are limitations for cleavable sites on targets. In some cases, the cleavable triplet sequence, NUX (N, any base; X, A, C, and U) (Shimayama *et al.*, 1995), is not available at a suitable position on the target. One situation involves chimeric mRNAs that are seen in various cases. As a well known example, the *BCR-ABL* chimeric mRNA is generated from reciprocal chromosomal translocation. Since the abnormal *BCR-ABL* chimeric mRNA causes chronic myelogenous leukemia (CML) (Groffen *et al.*, 1984), and is tumor-specific and pathogenetically important, it is obvious target for nucleic acids therapeutics as a paradigm (Shtivelman *et al.*, 1986). Despite the fact, because of the absence of NUX sequences proximal to the chimeric junction, conventional ribozymes failed to distinguish the chimeric mRNA from the normal mRNA (Kuwabara *et al.*, 1997; 1998b; Warashina *et al.*, 1999b). In designing ribozymes that might cleave chimeric mRNA, we must be sure to avoid cleavage of the normal mRNA, which shares partially the identical sequence with abnormal one.

As mentioned above, maxizymes can bind to two different target sites. Then, one can take advantage of the two substrate-binding regions to design a maxizyme with one substrate-binding region for the abnormal junction sequence and the second binding region for an efficient cleavage site even the site is distant from the junction. One substrate-binding site functions as the "eye (sensor)" to discriminate the chimeric mRNA, while the other serves as the "scissor" to actually cleave the target (Fig. 3c). The activity of the dimeric maxizyme was controlled allosterically by introducing sensor arms such that only after the binding with the real target (ex. abnormal *BCR-ABL* mRNA) can the maxizyme form a cavity that captures the Mg^{2+} ions. Since maxizymes, as well as hammerhead ribozymes, are metalloenzymes, their activity depends on the presence or absence of the correct formation of the Mg^{2+} ion-binding pocket (Kuwabara *et al.*, 1998b).

While, in the presence of normal mRNA (ex. normal *ABL* mRNA), which should not be cleaved, the structure of the catalytic core crumbles, so that the maxizyme becomes to be inactive (Fig. 3c). In addition, if the maxizyme binds to the cleavage site without the binding of sensor arms to abnormal or normal junction sequences, maxizyme structure remains also in inactive because the capture of the magnesium ion is not supported (Fig. 3c). In this maxizyme system, the *BCR-ABL* sequence is regarded as an activator for the allosteric enzyme, and the *ABL* sequence works as a rigorous inhibitor for the catalysis (Fig. 3c). In the absence of these effectors, the maxizyme lacks any catalytic activity. Chemical and enzymatic probings revealed that conformational changes of the maxizyme really occurred depending on these effector sequences (Zhou *et al.*, 2000), and it was demonstrated that the maxizyme indeed cleaved only the abnormal chimeric mRNA specifically in vitro (Kuwabara *et al.*, 1998b).

Anti-tumor effects of the allosterically controllable maxizyme Evaluation of the maxizyme was conducted in cultured mammalian cells. For efficient formation of dimers in cells, the high-level expression system via the tRNA-promoter that was recognized by RNA polymerase III was utilized (Kuwabara *et al.*, 1998b; 1999; Koseki *et al.*, 1999). The conventional ribozyme, each monomer of the maxizyme, and

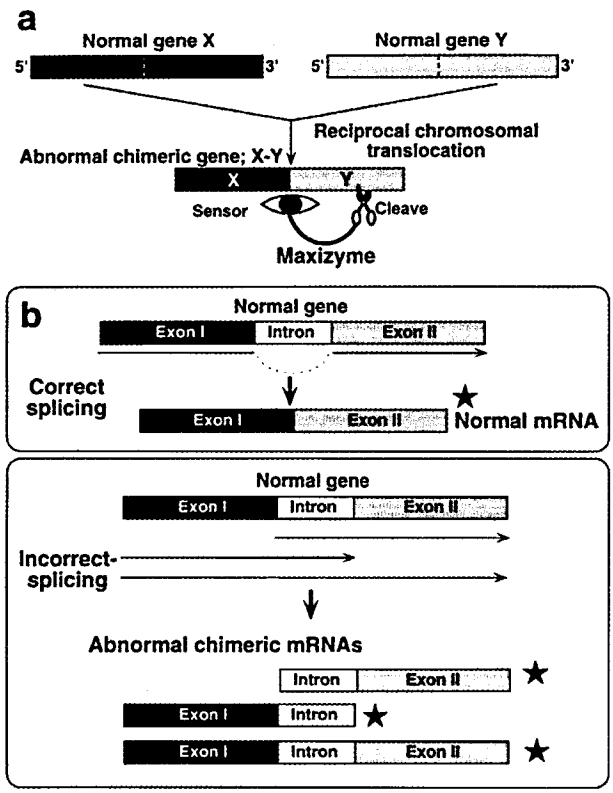

Fig. 4. Different types of transcripts that can be disrupted by a maxizyme. (a) A chimeric transcript can be recognized by sensor arms (indicated by "eye") at the junction and cleaved at a distant site by the maxizyme (the cleavage site is indicated by scissors). (b) Maxizymes can discriminate limited differences among various transcripts, and cleave specifically the correct target (marked with stars) in each case.

both monomers together were introduced into cells from patients with CML (Kuwabara *et al.*, 1998b). Analysis in cultured cells demonstrated that the active maxizyme was a heterodimer and that cleavage by the maxizyme, rather than an antisense effect, was responsible for the specific suppression of expression of the *BCR-ABL* mRNA by the allosteric function of the maxizyme. In addition, the maxizyme showed significantly higher activity than the wild-type ribozyme from which it was derived.

Furthermore, the anti-tumor effect of the maxizyme was subsequently examined in animals. All mice, without exception, that had been injected with CML patient cells died of diffuse leukemia (Tanabe *et al.*, 2000a). In marked contrast, when maxizyme-treated CML cells were injected, all mice remained disease-free. These results showed that the maxizyme apparently functioned not only in cultured cells but also in established model animals with exceptional efficacy and the maxizyme might be useful for gene therapy of CML.

Generality of the maxizyme technology The maxizyme is an allosteric ribozyme with biosensor functions that works in animals. By using this biosensor function, it has become possible to specifically inhibit the expression of gene of interest without affecting the normal mRNA in any case. By modulating the sequences of the sensor arms, the activity of the maxizyme can easily be adjusted. Therefore, the maxizyme technology is not limited only in one case of the disruption of abnormal chimeric gene in CML disease. Abnormal chimeric genes generated from reciprocal chromosomal translocation were observed frequently among several leukemia diseases (Fig. 4a). Maxizymes have successfully cleaved only abnormal targets, which are lacking a NUX cleavage site at the junction, of acute lymphoblastic leukemia (ALL) and acute promyelocytic leukemia (APL) without any damage of normal genes (Tanabe *et al.*, 2000b). In addition, abnormal chimeric mRNAs were generated as results of mis-splicings (Fig. 4b). In the case shown in Fig. 4b, the maxizyme can be designed against each possible transcript (indicated with stars) and should discriminate the target specifically from others by the allosteric function. Indeed, we have already constructed various maxizymes that target different chimeric genes, and each of them is very active and exquisitely specific (Kuwabara *et al.*, 1998b; Tanabe *et al.*, 2000b). Thus, maxizymes should be considered to be powerful gene-inactivating agents with allosteric functions to cleave 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 the potential utility in medical area.

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