

Comparison of the specificities and catalytic activities of hammerhead ribozymes and DNA enzymes with respect to the cleavage of *BCR–ABL* chimeric L6 (b2a2) mRNA

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ABSTRACT

With the eventual goal of developing a treatment for chronic myelogenous leukemia (CML), attempts have been made to design hammerhead ribozymes that can specifically cleave *BCR–ABL* fusion mRNA. In the case of L6 *BCR–ABL* fusion mRNA (b2a2 type; *BCR* exon 2 is fused to *ABL* exon 2), which has no effective cleavage sites for conventional hammerhead ribozymes near the *BCR–ABL* junction, it has proved very difficult to cleave the chimeric mRNA specifically. Several hammerhead ribozymes with relatively long junction-recognition sequences have poor substrate-specificity. Therefore, we explored the possibility of using newly selected DNA enzymes that can cleave RNA molecules with high activity to cleave L6 *BCR–ABL* fusion (b2a2) mRNA. In contrast to the results with the conventional ribozymes, the newly designed DNA enzymes, having higher flexibility for selection of cleavage sites, were able to cleave this chimeric RNA molecule specifically at sites close to the junction. Cleavage occurred only within the abnormal *BCR–ABL* mRNA, without any cleavage of the normal *ABL* or *BCR* mRNA. Thus, these chemically synthesized DNA enzymes seem to be potentially useful for application *in vivo*, especially for the treatment of CML, if we can develop exogenous delivery strategies.

INTRODUCTION

Hammerhead ribozymes are among the smallest catalytic RNAs. The sequence motif, with three duplex stems and a conserved 'core' of two non-helical segments that are responsible for self-cleavage (*cis* action), was first recognized in the satellite

RNAs of certain viruses (1). The *trans*-acting hammerhead ribozyme, which was designed by Uhlenbeck (2) and Haseloff and Gerlach (3), consists of an antisense section (stems I and III) and a catalytic domain with a flanking stem–loop II section (3). Such RNA motifs can cleave oligoribonucleotides at specific sites (most effectively at GUC) (4–8). Because of its small size and potential utility as an anti-virus agent, this ribozyme has been extensively investigated in terms of the mechanism of its action and possible applications *in vivo* (9–26). For such applications, it is clearly necessary to direct the ribozyme specifically to the cellular RNA target of interest.

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder of hematopoietic stem cells associated with the Philadelphia chromosome (27). The reciprocal chromosomal translocations t(9;22)(q34;q11) can be subdivided into two types, K28 translocations and L6 translocations (Fig. 1). They result in the formation of the *BCR–ABL* fusion gene which encodes two types of mRNA; b3a2 (consisting of *BCR* exon 3 and *ABL* exon 2) and b2a2 (consisting of the *BCR* exon 2 and *ABL* exon 2) (28–33). Both of these mRNAs are translated into a protein of 210 kDa (p210^{*BCR–ABL*}) which is unique to the malignant cell phenotype (34).

For the design of ribozymes that will disrupt chimeric RNAs, it is necessary to target the junction sequence. Otherwise, normal mRNAs that share part of the chimeric RNA sequence would also be cleaved by the ribozyme, with resultant damage to the host cells. In the case of the *BCR–ABL* chimeric RNA sequence b3a2, a potential ribozyme-cleavage site, a GUU triplet, is located 3 nucleotides (nt) upstream from the chimeric junction (Fig. 1). Therefore, a conventionally designed hammerhead ribozyme might be expected to cleave specifically the abnormal b3a2 mRNA generated from K28 translocations. Indeed, several such examples have been reported (35–44). By contrast, in the case of the b2a2 sequence, which results from L6 translocations, as well

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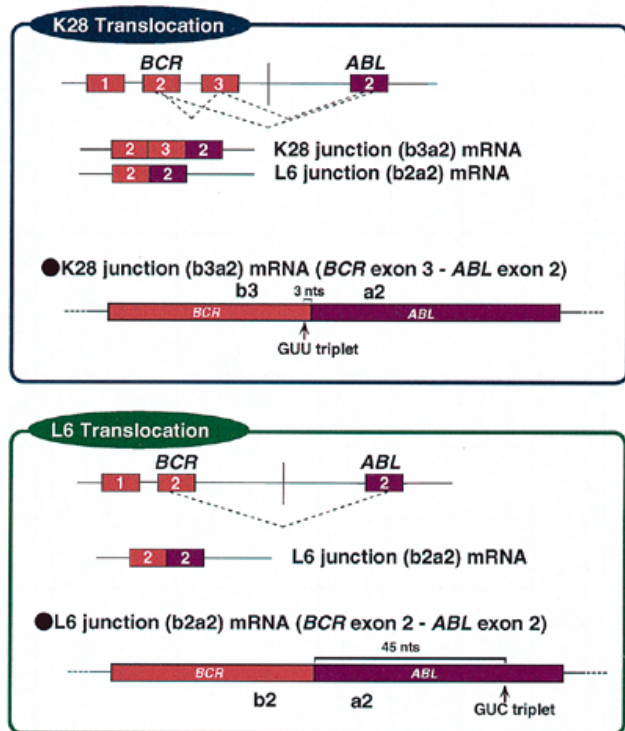


Figure 1. *BCR-ABL* translocations and fusion mRNAs. The two types of chromosomal translocation [K28-type (upper panel) and L6-type (lower panel)] that are associated with CML and the corresponding fusion mRNAs are depicted. Boxes in red represent *BCR* exons and boxes in purple represent *ABL* exon 2. Dotted lines connecting *BCR* and *ABL* exons indicate alternative splicing pathways.

as some K28 translocations (Fig. 1), there are no triplet sequences that are potentially cleavable by hammerhead ribozymes within 2 or 3 nt of the *BCR-ABL* junction. In the sequence of b2a2, the closest ribozyme-cleavage sites in the vicinity of the *BCR-ABL* junction are located 7, 8, 9 and 19 nt away from the junction (Fig. 2). A GUC triplet, which is generally most susceptible to cleavage by hammerhead ribozymes, is also located 45 nt from the junction. In designing ribozymes that might cleave b2a2 mRNA, we must be sure to avoid cleavage of the normal *ABL* mRNA itself. Previous attempts have involved a combination of a long antisense arm and the ribozyme sequence (45,46).

In a recent study, Santoro and Joyce successfully selected DNA enzymes, that can cleave RNA molecules with any sequence, by using an *in vitro* selection procedure (47). Joyce's DNA enzymes are rather similar to the conventional hammerhead ribozyme. They consist of a catalytic domain of 15 deoxynucleotides and Mg^{2+} ions are necessary for catalytic activity, as in the case of hammerhead ribozymes, which are recognized as metalloenzymes (48–64). The catalytic domain is flanked by two substrate-recognition domains of 7 or 8 deoxyribonucleotides each, and the RNA substrate is bound through Watson–Crick base pairing (Fig. 2). Joyce's DNA enzymes can be divided into two types. Type I DNA enzymes can cleave an RNA sequence at a phosphodiester bond located between an A residue and a G residue (DNA enzymes 1 and 2 in Fig. 2). The catalytic domain

consists of a 4 nt loop by the cleavage site and a stem–loop region that resembles the stem–loop II region of the hammerhead ribozyme. However, the former stem–loop region is essential for catalysis (47). The type II DNA enzymes can cleave an RNA sequence at a phosphodiester bond located between a purine and pyrimidine residue. In this case, the catalytic domain consists of 15 nt (DNA enzyme 3 in Fig. 2). Such DNA enzymes can be expected to cleave almost any target RNA substrate. Indeed, Santoro and Joyce demonstrated that their DNA enzymes could cleave HIV-1 mRNAs (47).

Since we were interested in cleaving b2a2 mRNA and since several sites of potential cleavage by Joyce's DNA enzymes are located within 3 nt of the *BCR-ABL* junction, we compared the specificity and catalytic activity of conventional hammerhead ribozymes and Joyce's DNA enzymes with respect to the cleavage of *BCR-ABL* chimeric L6 (b2a2) mRNA.

MATERIALS AND METHODS

Synthesis of ribozymes and DNA enzymes

Ribozymes (41mer Rz and Rz37) and DNA enzymes were chemically synthesized on a DNA–RNA synthesizer [model 394; Applied Biosystems, Division of Perkin Elmer Co. (ABI), Foster City, CA]. Reagents for RNA synthesis were purchased either from ABI or Glen Research (VA). Oligonucleotides were purified as described in the user bulletin from ABI (no. 53; 1989) with minor modifications. Further purification was based on polyacrylamide gel electrophoresis as described previously (7). The sequence of the 41mer Rz was identical to that used in previous studies (45). Rz37, which targets the GUC triplet located 45 nt 3' of the *BCR-ABL* junction had the following sequence: 5'-CAC UCA CUG AUG AGG CCG AAA GGC CGA AAC CCU GAG G-3'. Since Rz37 was the only new ribozyme sequence used in this study, we used different nomenclature for this ribozyme (the sequences of other ribozymes such as 41mer Rz, 52mer Rz and 81mer Rz were taken either from ref. 45 or from ref. 46).

Preparation of ribozymes by transcription

DNA templates for ribozymes (52mer Rz and 81mer Rz) that were synthesized chemically contained the T7 promoter. Downstream of the promoter sequence, we inserted no residues, such as two or three G residues for higher efficiency of transcription, so that the sequences of the products were identical to those reported in the literature (45,46). Products of PCR were gel-purified. T7 transcription *in vitro* and gel-electrophoretic purification of the 52mer Rz and 81mer Rz were performed as described elsewhere (7).

Preparation of target substrates, namely, *ABL*, *BCR-ABL* and *BCR* mRNAs, by transcription

DNA templates for L6 *BCR-ABL* substrate RNA and for both the normal *ABL* and *BCR* substrate RNAs were synthesized chemically. The DNA oligodeoxynucleotide template for L6 *BCR-ABL* substrate RNA consisted of the sequence from 63 nt 5' of the *BCR-ABL* junction to 58 nt 3' of the *BCR-ABL* junction. The region of the DNA oligodeoxynucleotide template for the normal *ABL* substrate RNA extended from position 192 to 283 of normal *ABL* cDNA (31–33). The DNA template for normal *BCR* substrate RNA extended from position 3234 to 3363 of normal *BCR* cDNA (31–33). Primers were also synthesized for each

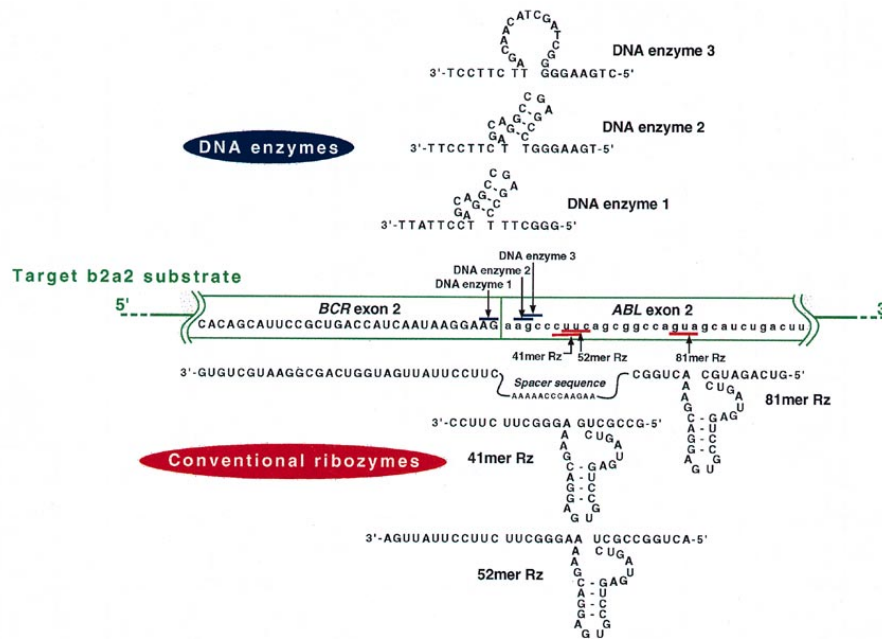


Figure 2. Nucleotide sequences of the conventional antisense-type ribozymes and DNA enzymes targeted to the L6 *BCR-ABL* (b2a2) substrate. The sequence of L6 *BCR-ABL* near the junction is expanded. The *BCR* exon 2 sequence near the junction is depicted by capital letters and that of the *ABL* exon 2 sequence is shown in lowercase letters. The sites of cleavage by antisense-type ribozymes (81mer Rz, 41mer Rz and 52mer Rz) are indicated by red lines and those of DNA enzymes by blue lines. The sequences of 41mer Rz and 81mer Rz were taken from ref. 45. The sequence of 52mer Rz was taken from ref. 46.

template, and each sense strand contained the T7 promoter. The sequences of the 5' and the 3' primers for L6 *BCR-ABL* substrate RNA were 5'-TAA TAC GAC TCA CTA TAG GGA CAA CTC GTG TGT GAA ACT C-3' and 5'-GCG GCT TCA CTC AGA CCC TGA GGC T-3'. The sequences of the 5' and the 3' primers for the normal *ABL* substrate RNA were 5'-TAA TAC GAC TCA CTA TAG GGC AGA TGC TGA CCA ACT CGT G-3' and 5'-ATC CAG TGG CTG AGT GGA CGA TGA C-3'. The products of PCR were gel-purified. T7 transcription *in vitro* and gel-electrophoretic purification of the *ABL*, *BCR-ABL* and *BCR* mRNA substrates were performed as described elsewhere (7).

Qualitative assays of ribozyme and DNA enzyme activities

Assays of ribozyme and DNA enzyme activities were performed, in 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0), under enzyme-saturating (single-turnover) conditions at 37°C, with incubation for 60 min (Fig. 4A and Fig. 5). The substrates were labeled with $[\gamma\text{-}^{32}P]\text{ATP}$ by T4 polynucleotide kinase (Takara Shuzo, Kyoto, Japan). Each enzyme (ribozyme or DNA enzyme) was incubated at 1 μM with 2 nM 5'- ^{32}P -labeled substrate. Furthermore, we also re-examined the activities and specificities of conventionally designed hammerhead ribozymes (Fig. 4B and C) under conditions used in previous studies (45,46). Specifically, reactions shown in Figure 4 were carried out in 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.5) with 1 μM each of the ribozyme and 1 μM substrate at 37°C, with incubation for 6 h (45). Reactions shown in Figure 4C were carried out in 20 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0) with 50 nM each of the

ribozyme and 10 nM substrate at 37°C, with incubation for 2 h (46). Reactions were usually initiated by the addition of $MgCl_2$ to a buffered solution that contained either the ribozyme or the DNA enzyme together with the substrate and each resultant mixture was then incubated at 37°C.

Kinetic analysis

Kinetic measurements for DNA enzymes were performed either with a long substrate (*BCR-ABL* 121mer substrate) or with a short 21mer substrate (S21) that had the following sequence; 5'-AAU AAG GAA GAA GCC CUU CAG-3'. The latter substrate, S21, contained three cleavage sites, each of which can be targeted by a respective DNA enzyme used in this study. As a control, kinetic measurements for a conventional hammerhead ribozyme (Rz37) were also performed either with a long substrate (*BCR-ABL* 121mer substrate) or with a short 16mer substrate (S16) that had the following sequence; 5'-CCU CAG GGU CUG AGU G-3'. In general, reaction rates were measured in 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0), under enzyme-saturating (single-turnover) conditions at 37°C. In the case of Rz37 with the short S16 substrate, because of the high cleavage activity, reaction rates were measured at pH 7.0 in 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.0), under single-turnover conditions at 37°C (Table 1).

Reactions were usually initiated by the addition of $MgCl_2$ to a buffered solution that contained either the ribozyme or the DNA enzyme together with the substrate, and each resultant mixture was then incubated at 37°C. In the case of the long *BCR-ABL* 121mer substrate (Fig. 6 and k_{obs} in Table 1), kinetic measurements were made under conditions where all the available substrate was expected to form a Michaelis-Menten complex, with high concentrations of the ribozyme or the DNA enzyme (from 10 to 20 μM).

Table 1. Kinetic parameters for the cleavage of *BCR-ABL* mRNA^a

Enzyme	S21		S16		<i>BCR-ABL</i> (121mer)
	k_{cat} (min ⁻¹)	K_M (nM)	k_{cat} (min ⁻¹)	K_M (nM)	k_{obs} (min ⁻¹)
DNA enzyme 1	0.012	83			0.0049
DNA enzyme 2	0.003	570			0.0029
DNA enzyme 3	0.012	36			0.0073
Rz37			0.13 ^a	190 ^a	>0.5

^aRate constants were measured, in 50 mM Tris-HCl (pH 8.0) and 25 mM MgCl₂ under enzyme saturating (single-turnover) conditions at 37°C, except for the Rz37-mediated cleavage of the S16 substrate where reactions were performed in 50 mM Tris-HCl (pH 7.0) because of the high cleavage activity of Rz37. In the cases of *BCR-ABL* 121mer substrate, kinetic measurements were made under conditions where all the substrate was expected to form a Michaelis-Menten complex, with high concentrations of enzymes (from 10 to 20 μM). Rate constants are averages from two sets of experiments.

Reactions were stopped at intervals by removal of aliquots from the reaction mixture 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. The substrate and the products of the reaction were separated by electrophoresis on an 8% 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). Cleavage rates were obtained from the slopes of the curves for the time-course of reactions at the initial stage, and K_M and k_{cat} were calculated from Eadie-Hofstee plots. In the determination of K_M and k_{cat} , between four and six different concentrations of enzymes, spanning the K_M , were used and the calculated values are summarized in Table 1. Potential errors in these values were found to be 10% from results of duplicate experiments (two sets of Eadie-Hofstee plots).

RESULTS AND DISCUSSION

Design of catalytic molecules and selection of their target sites

The design of hammerhead ribozymes and their target sites for the specific cleavage of b2a2 mRNA were based on previously published results (45,46). Long antisense sequences of ~10–30 nt in length, which could bind to and cover the junction region for some distance from the cleavage sites, were connected to binding sites of hammerhead ribozymes (81mer, 41mer and 52mer Rz's in Fig. 2). The lengths of annealing arms are important for the activity of ribozymes because they influence the efficiency, as well as the specificity, of the cleavage reaction (26,42). In the case of a ribozyme that is directed against two non-contiguous sequences, the specificity is particularly important if we are to avoid non-specific cleavage of normal mRNAs. Among the conventional ribozymes depicted in Figure 2, which were designed to cleave L6 *BCR-ABL* chimeric mRNA specifically, the 52mer Rz and the 41mer Rz had long binding arms, in the stem III region, of 20 and 12 nt, respectively. In the case of the 81mer Rz, the binding arms were 50 nt in length in the stem III region and were connected to the ribozyme sequence by a 13 nt spacer sequence that was non-complementary to the substrate, to achieve greater flexibility of binding (45). The 52mer Rz was designed to cleave the L6 *BCR-ABL* mRNA at the UUC triplet located 9 nt 3' of the junction

(46). The 41mer Rz was designed to cleave the substrate at the CUU triplet located 8 nt 3' of the junction. The 81mer Rz was designed to cleave the substrate at the GUA triplet located 19 nt 3' of the junction. According to a published report (45), the 81mer and 41mer Rz's should have enhanced specificity for the chimeric b2a2 mRNA substrate. However, according to other studies (65,66), it seems that hammerhead ribozymes have cleavage ability even if the binding arm is as little as 3 nt in length. As can be seen from Figure 2, the binding region of these antisense-type ribozymes to the normal *ABL* mRNA sequence consisted of at least 6 bp. Therefore, we could not exclude the possibility that such ribozymes might bind non-specifically to and cleave the normal *ABL* mRNA just as they specifically cleave the *BCR-ABL* (b2a2) mRNA. Moreover, longer substrate-binding arms might lower the rate of dissociation from the substrate, with a resultant reduction in the ribozyme-turnover rate. Therefore, we synthesized ribozymes (81mer, 41mer and 52mer Rz's in Fig. 2) that were identical to those in the literature (45,46) and re-examined their specificities. However, the substrates (92mer *ABL* substrate and 121mer *BCR-ABL* substrate) used in this study are shorter in length than those used in previous studies (45,46). As described in Materials and Methods, the 41mer Rz was synthesized chemically and the 52mer and 81mer Rz's were generated by transcription from the corresponding DNA templates.

The chimeric b2a2 mRNA substrate of 121 nt in length (Fig. 3), the normal *ABL* substrate of 92 nt and the *BCR* substrate of 130 nt were also generated by transcription. As a control, for the comparison of kinetic parameters, we also synthesized chemically Rz37, which can cleave the GUC triplet located 45 nt downstream from the *BCR-ABL* junction (Fig. 3). We note here that, since the cleavage site of Rz37 was located far from the *BCR-ABL* junction, Rz37 could cleave both normal *ABL* mRNA and chimeric *BCR-ABL* mRNA.

Examination of the *BCR-ABL* junction (b2a2 substrate) revealed the presence of several potential target sites, within 3 nt of the junction, for Joyce's DNA enzymes (47). For example, the first AG sequence is located 1 nt 5' of the junction. A second AG sequence is located 2 nt 3' of the junction, and a GC sequence is located 3 nt 3' of the junction. Therefore, we designed three kinds of DNA enzyme, each of which targeted one of these cleavage sites (Fig. 2). For the AG cleavage sites, we used type I DNA enzymes (DNA enzymes 1 and 2). A type II DNA enzyme was used for the GC cleavage site (DNA enzyme 3). DNA enzyme 1 should cleave the *BCR* region of the chimeric *BCR-ABL* mRNA,

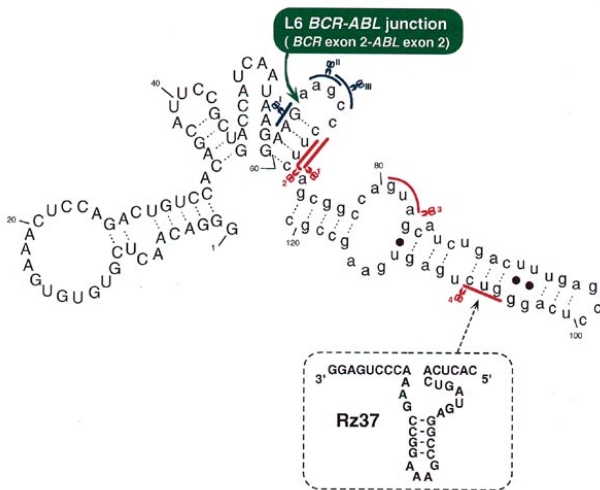


Figure 3. Secondary structure of the *BCR-ABL* (b2a2) substrate (121mer) used in this study, as predicted by a computer program (MulFold). The *BCR* region of the chimeric *BCR-ABL* (b2a2) substrate is given in capital letters and the *ABL* region is given in lowercase letters. Cleavage sites of ribozymes (I, 41mer Rz; 2, 52mer Rz; 3, 81mer Rz; 4, Rz37) and DNA enzymes (I, DNA enzyme 1; II, DNA enzyme 2; III, DNA enzyme 3) are indicated by 'scissors'. The sequence of Rz37 at its cleavage site is also depicted.

whereas DNA enzymes 2 and 3 should cleave the *ABL* region of the chimeric mRNA.

Specificity of cleavage of the chimeric *BCR-ABL* L6 (b2a2) substrate

In order to examine the specificity of cleavage reactions catalyzed either by conventional ribozymes or by Joyce's DNA enzymes (45–47), we examined three types of RNA substrate (Figs 4 and 5), namely, the normal *ABL* substrate, the chimeric *BCR-ABL* substrate and the normal *BCR* substrate, with lengths of 92, 121 and 130 nt, respectively. Enzymes with high specificity should cleave only the chimeric *BCR-ABL* substrate. Kinetic measurements shown in Figures 4A, 5 and 6 (and also measurements of most of the k_{cat} and k_{obs} values listed in Table 1) were made in 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0), under enzyme-saturating (single-turnover) conditions at 37°C, namely our standard conditions for kinetic measurements (56).

The results for the conventionally designed ribozymes are shown in Figure 4A. As expected, all the conventional ribozymes cleaved the *BCR-ABL* substrate at the anticipated sites. However, in contrast to expectations based on previous reports, not only the control Rz37 but also the antisense-type ribozymes (45,46) cleaved the normal *ABL* substrate within 1 h under our standard conditions for kinetic measurements (Condition 1 in Table 2). Moreover, as can be seen from Figure 4A, the amounts of cleavage products obtained from the normal *ABL* mRNA with each ribozyme were almost the same as those obtained from the chimeric *BCR-ABL* substrate, indicating that these conventional ribozymes, with their relatively long antisense arms, recognized not only the abnormal *BCR-ABL* mRNA but also the normal *ABL* mRNA as substrates. Therefore, non-specific cleavage of normal *ABL* mRNA could not be avoided when we used conventionally designed ribozymes. However, since our reaction conditions were

different from the reported conditions (45,46), we re-examined the cleavage reactions under the previously reported conditions. The results are shown in Figure 4B and C, and the extent of cleavage was determined by quantitation of radioactivity by an Image Analyzer and they are summarized under % 'Cleavage' in Table 2. Conditions 2 and 3 correspond to the reaction conditions used in references 45 and 46, respectively. To our surprise, as can be seen from Figure 4B and C and Table 2, we were unable to reproduce the previously reported specificity with our substrates.

In previous studies on these long antisense-type ribozymes, one part of the target site was designed to be accessible for annealing and served to direct ribozyme nucleation, while the other part recognized the cleavage triplet in the vicinity of the *BCR-ABL* junction, where specific cleavage of the hybrid mRNA occurred. We note that, in all cases, these conventional Rz's have regions of complementary binding to the normal *ABL* mRNA sequences of at least 6–8 nt. Previous studies of hammerhead ribozymes (65,66) demonstrated that cleavage of the substrate could occur when one of the substrate-binding arms was 3 nt long. Thus, in some cases, one would not expect substrate specificity from the conventionally designed ribozymes shown in Figure 2. Indeed, when we used substrates that were shorter in length than those used in previous studies [the conventionally designed ribozymes demonstrated high specificity when those relatively long substrates were used (45,46)], we were unable to observe meaningful specificity.

In terms of substrate specificity, Joyce's DNA enzymes, as depicted in Figure 2, were expected to show high specificity for the L6 *BCR-ABL* substrate because all the target sites were located near the chimeric *BCR-ABL* junction (within 3 nt of the junction). DNA enzyme 1 was designed to cleave the *BCR* region of the chimeric *BCR-ABL* mRNA, whereas DNA enzymes 2 and 3 were designed to cleave the *ABL* region of the chimeric mRNA. Therefore, as control substrates, both normal *ABL* mRNA and *BCR* mRNA were used in addition to the chimeric *BCR-ABL* mRNA (Fig. 5). The specificity of the newly designed DNA enzymes for the chimeric *BCR-ABL* substrate was tested by incubating the DNA enzymes with either the chimeric *BCR-ABL* substrate or the normal *ABL* or *BCR* substrate. As demonstrated in Figure 5, all the DNA enzymes cleaved the L6 *BCR-ABL* substrate at the anticipated cleavage site, producing products of the expected sizes. No products of cleavage of the normal *ABL* or the *BCR* substrate were detected in any of these reactions, demonstrating the expected high substrate-specificity of these DNA enzymes.

Determination of kinetic parameters

To identify the cleavage activity of the DNA enzymes, we determined the kinetic parameters for the cleavage of the *BCR-ABL* 121mer substrate under single-turnover conditions. The results (determination of k_{obs}) are summarized in Table 1 and typical time-courses are shown in Figure 6. In terms of kinetic parameters (k_{obs}), at least for this particular substrate, DNA enzyme 3 was more active than other DNA enzymes, although target sites differed slightly among enzymes. In order to characterize in further detail the properties of the DNA enzymes, we also determined the kinetic parameters for the cleavage of a short 21mer substrate (S21) by the DNA enzymes at pH 8.0 and also for the cleavage of a short 16mer substrate (S16) by Rz37 at pH 7.0. In the case of Rz37 with the short S16 substrate, because of the high cleavage activity, reactions were carried out at pH 7.0 rather than at pH 8.0 (Table 1).

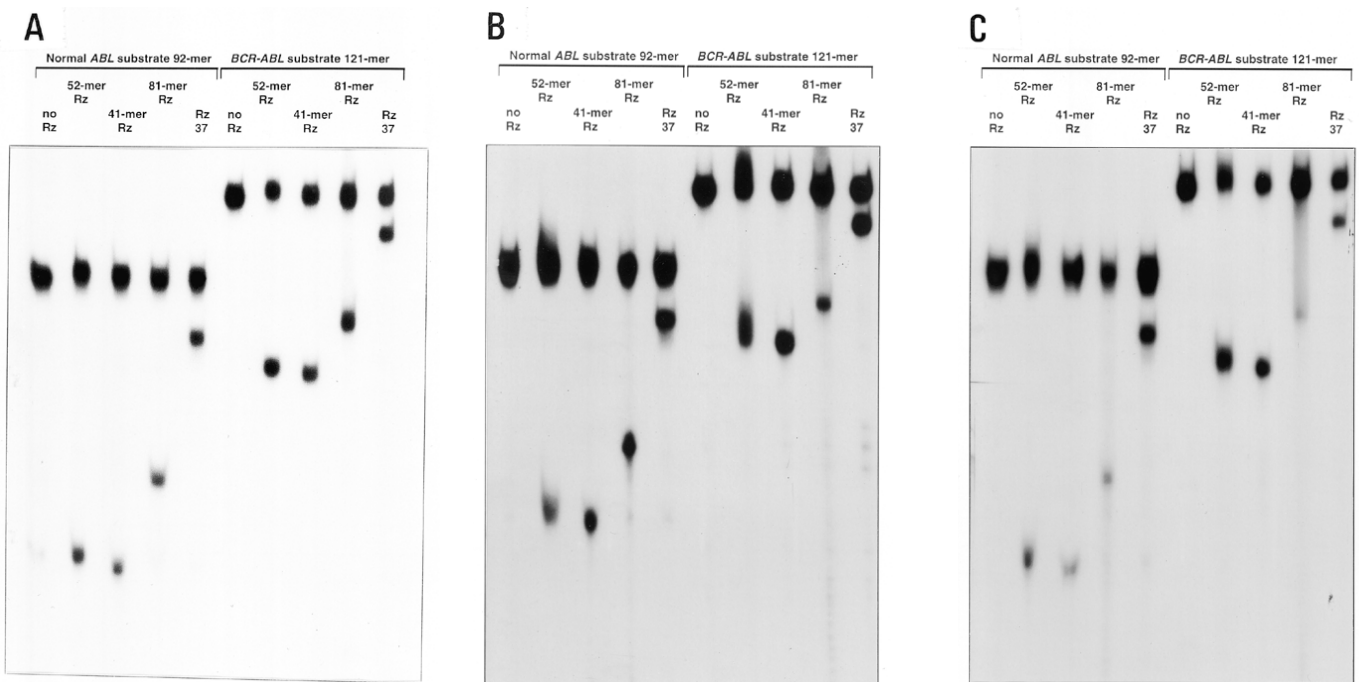


Figure 4. Autoradiogram after gel electrophoresis showing the non-specific cleavage of chimeric *BCR-ABL* mRNA, as well as of normal *ABL* mRNA, by conventional ribozymes. Specificity was examined with the normal *ABL* substrate (92mer) and the chimeric *BCR-ABL* substrate (121mer). (A) Each ribozyme (1 μ M) and 2 nM $5'$ - 32 P-labeled substrate were incubated at 37°C for 60 min in a solution that contained 50 mM Tris-HCl (pH 8.0) and 25 mM $MgCl_2$. (B) Each ribozyme (1 μ M) and 1 μ M substrate were incubated at 37°C for 6 h in a solution that contained 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$. (C) 50 nM each of the ribozyme and 10 nM substrate were incubated at 37°C for 2 h in a solution that contained 50 mM Tris-HCl (pH 8.0) and 20 mM $MgCl_2$. Then, each reaction mixture was subjected to electrophoresis on an 8% polyacrylamide/7 M urea gel. Cleavage products from the normal *ABL* substrate (92mer) were as follows. Non-specific cleavage, at the UUC triplet located 9 nt 3' of the junction, by the 52mer Rz generated a 32 P-labeled 5'-fragment of 43 nt in length. Similarly, non-specific cleavage, at the CUU triplet located 8 nt 3' of the junction, by the 41mer Rz generated a visible fragment of 42 nt. Non-specific cleavage, at GUA located 19 nt 3' of the junction, by the 81mer Rz generated a fragment of 54 nt. Cleavage at GUC located 45 nt 3' of the junction, by Rz37 generated a fragment of 79 nt. Cleavage products from the *BCR-ABL* substrate (121mer) were as follows. Cleavage by the 52mer Rz generated a visible 5'-fragment of 72 nt in length. Similarly, cleavage by the 41mer Rz generated a fragment of 71 nt. Cleavage by the 81mer Rz generated a fragment of 83 nt. Cleavage by the Rz37 generated a fragment of 108 nt in length.

Table 2. Activities and specificities of conventional ribozymes for the cleavage of *BCR-ABL* mRNA^a

Enzymes	Condition 1			Condition 2			Condition 3		
	% Cleavage	Product ratio		% Cleavage	Product ratio		% Cleavage	Product ratio	
	<i>ABL</i>	<i>BCR-ABL</i>	<i>ABL/BCR-ABL</i>	<i>ABL</i>	<i>BCR-ABL</i>	<i>ABL/BCR-ABL</i>	<i>ABL</i>	<i>BCR-ABL</i>	<i>ABL/BCR-ABL</i>
52mer Rz	32	58	0.55	16	28	0.58	19	43	0.44
41mer Rz	26	46	0.57	18	35	0.52	9.2	36	0.27
81mer Rz	37	40	0.92	23	9.1	2.6	13.7	8.2	1.7
Rz37	36	44	0.82	29	35	0.83	21	29	0.72
Reaction conditions									
pH	8.0			7.5			8.0		
Mg^{2+} (mM)	25			10			20		
Enzyme (μ M)	1.0			1.0			0.05		
Substrate (μ M)	< 0.0020			1.0			0.01		
Incubation (h)	1.0			6.0			2.0		

^aThree different conditions were used to examine activities and specificities of conventional ribozymes. Condition 1 was also used to measure kinetic parameters listed in Table 1. Conditions 2 (45) and 3 (46) were the reaction conditions used in previous studies.

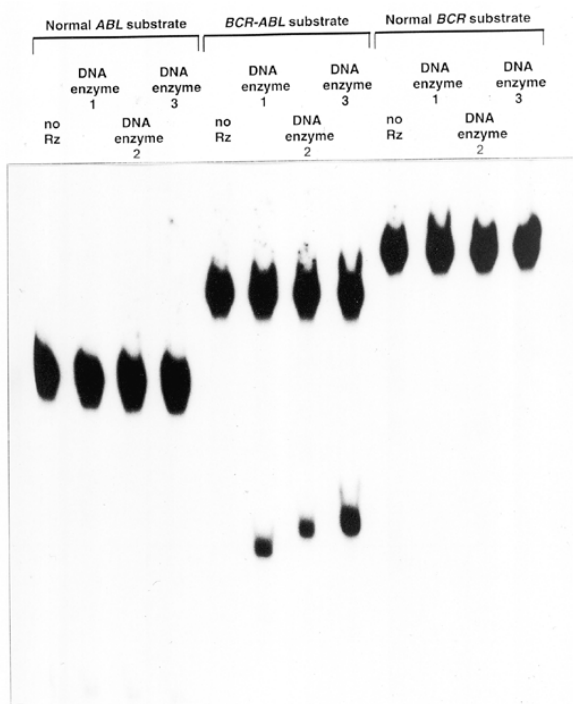


Figure 5. Gel electrophoresis showing cleavage by DNA enzymes. Specificity in cleavage by DNA enzymes was examined by using the normal *ABL* substrate (92mer), the normal *BCR* substrate (130mer) and the chimeric *BCR-ABL* substrate (121mer). Each DNA enzyme (1 μ M) and 2 nM $5'$ - 32 P-labeled substrate were incubated at 37°C for 60 min in a solution that contained 50 mM Tris-HCl (pH 8.0) and 25 mM MgCl₂ and then subjected to electrophoresis on an 8% polyacrylamide/7M urea gel. Cleavage of the *BCR-ABL* substrate, at the AG sequence located 1 nt 5' of the junction, by DNA enzyme 1 generated a visible 5' fragment of 62 nt in length. Similarly, cleavage at the second AG sequence, located 2 nt 3' of the junction, by DNA enzyme 2 generated a fragment of 65 nt. Cleavage at the GC sequence located 3 nt 3' of the junction by DNA enzyme 3 generated a fragment of 66 nt. No cleavage of the normal *ABL* and *BCR* substrates occurred.

Comparison of kinetic parameters revealed that, at least for the particular substrates used in this study, the hammerhead ribozyme, Rz37, was more active than the DNA enzymes (Table 1), although target sites differed among enzymes. It is generally accepted that long RNA transcripts are cleaved less efficiently by ribozymes, because of their higher ordered structures, than corresponding short synthetic oligoribonucleotide substrates (13,67,68). This was also true in our case (Table 1). In terms of k_{cat}/K_M , DNA enzymes were shown previously to be more powerful than hammerhead ribozymes in the cleavage of HIV-1 mRNA (47). However, in our case, Rz37 was more active than the DNA enzymes, partly because we did not make any attempts to optimize the length of the substrate-binding arms [Joyce claims it is important to optimize them in order to obtain optimum activities of DNA enzymes (47)].

Potential gene therapy for treatment of CML

The specific association of nucleic acid-based drugs, such as ribozymes and Joyce's DNA enzymes, with their targets via base pairing and subsequent cleavage of the RNA substrate suggest that these catalytic molecules might be useful for gene therapy.

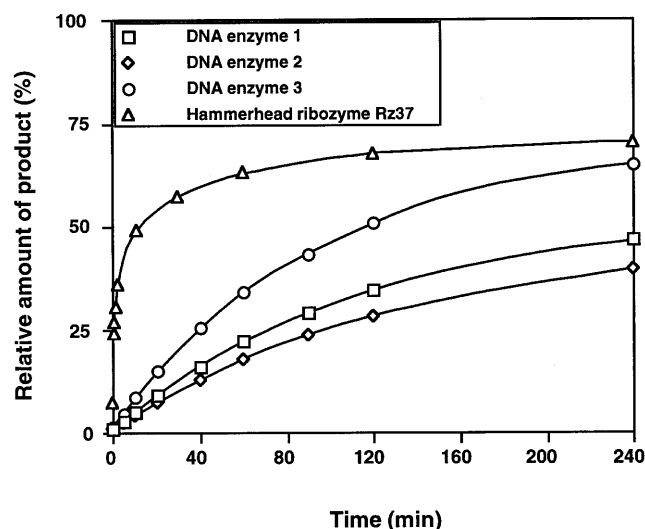


Figure 6. Time courses for DNA enzyme-mediated and ribozyme-mediated cleavage reactions. Relative amounts of cleavage products (%) are plotted versus time. Calculated values of k_{obs} are shown in Table 1. Symbols: \square , DNA enzyme 1; \diamond , DNA enzyme 2; \circ , DNA enzyme 3; Δ , Rz 37.

There are basically two ways to introduce ribozymes into cells. One such technique is a drug-delivery system (DDS) in which a chemically synthesized ribozyme (or DNA enzyme) is encapsulated in liposomes or other related compounds and delivered to target cells. Another way to introduce ribozymes into cells is by transcription from the corresponding DNA template (gene therapy). Current gene therapy technology is limited primarily by the necessity for *ex vivo* manipulations of target tissues, namely, target cells must be removed from the body, engineered and returned. Therefore, the limitations that determine the genetic diseases that can potentially be treated are directly linked to the limitations of current cell biology (69).

For the treatment of CML by nucleic acid drugs, in particular in the case of the L6 translocations on which we focused in this study, a conventional ribozyme may not be the best choice because of the lack of substrate specificity depending on the higher ordered structure of the target molecule. However, Joyce's DNA enzymes, despite their apparently lower cleavage activities, at least with the substrates used in this study, are suitable molecules for DDS, because DNA molecules are more stable than RNA molecules *in vivo*. The higher stability *in vivo* of DNA enzymes should, in principle, counteract the apparent lower activity of the DNA enzymes because longer incubation resulted in a similar extent of cleavage of the chimeric *BCR-ABL* mRNA (Fig. 6). Since DNA enzymes are easier to synthesize, easier to handle and more stable *in vivo* than ribozymes, we anticipate that all synthetic, catalytic nucleic acid drugs for DDS will turn out to be DNA enzymes, with additional modifications for higher stability and for higher affinity for their target molecules (70).

Nevertheless, current gene therapy technology is suited for ribozymes more than for DNA enzymes, because the former but not the latter can be transcribed *in vivo*. Since the conventionally designed ribozyme may not be the best choice for the treatment of CML, especially in the case of L6 translocations, we are at present exploring the possibility of using our dimeric minizymes (71-74) for the treatment of CML.

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