

Extremely high and specific activity of DNA enzymes in cells with a Philadelphia chromosome

Masaki Warashina, Tomoko Kuwabara, Yuka Nakamatsu and Kazunari Taira

Background: Chronic myelogenous leukemia (CML) results from chromosome 22 translocations (the Philadelphia chromosome) that creates *BCR-ABL* fusion genes, which encode two abnormal mRNAs (b3a2 and b2a2). Various attempts to design antisense oligonucleotides that specifically cleave abnormal L6 *BCR-ABL* fusion mRNA have not been successful. Because b2a2 mRNA cannot be effectively cleaved by hammerhead ribozymes near the *BCR-ABL* junction, it has proved very difficult to engineer specific cleavage of this chimeric mRNA. Nonspecific effects associated with using antisense molecules make the use of such antisense molecules questionable.

Results: The usefulness of DNA enzymes in specifically suppressing expression of L6 *BCR-ABL* mRNA in mammalian cells is demonstrated. Although the efficacy of DNA enzymes with natural linkages decreased 12 hours after transfection, partially modified DNA enzymes, with either phosphorothioate or 2'-O-methyl groups at both their 5' and 3' ends, remained active for much longer times in mammalian cells. Moreover, the DNA enzyme with only 2'-O-methyl modifications was also highly specific for abnormal mRNA.

Conclusions: DNA enzymes with 2'-O-methyl modifications are potentially useful as gene-inactivating agents in the treatment of diseases such as CML. In contrast to conventional antisense DNAs, some of the DNA enzymes used in this study were highly specific and cleaved only abnormal *BCR-ABL* mRNA.

Addresses: National Institute for Advanced Interdisciplinary Research, AIST, MITI, Tsukuba Science City 305-8562, Japan. National Institute of Bioscience and Human Technology, AIST, MITI, Tsukuba Science City 305-8566, Japan. Institute of Applied Biochemistry, University of Tsukuba, Tennoudai 1-1-1, Tsukuba Science City 305-8572, Japan.

Correspondence: Kazunari Taira
E-mail: taira@nair.go.jp

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Introduction

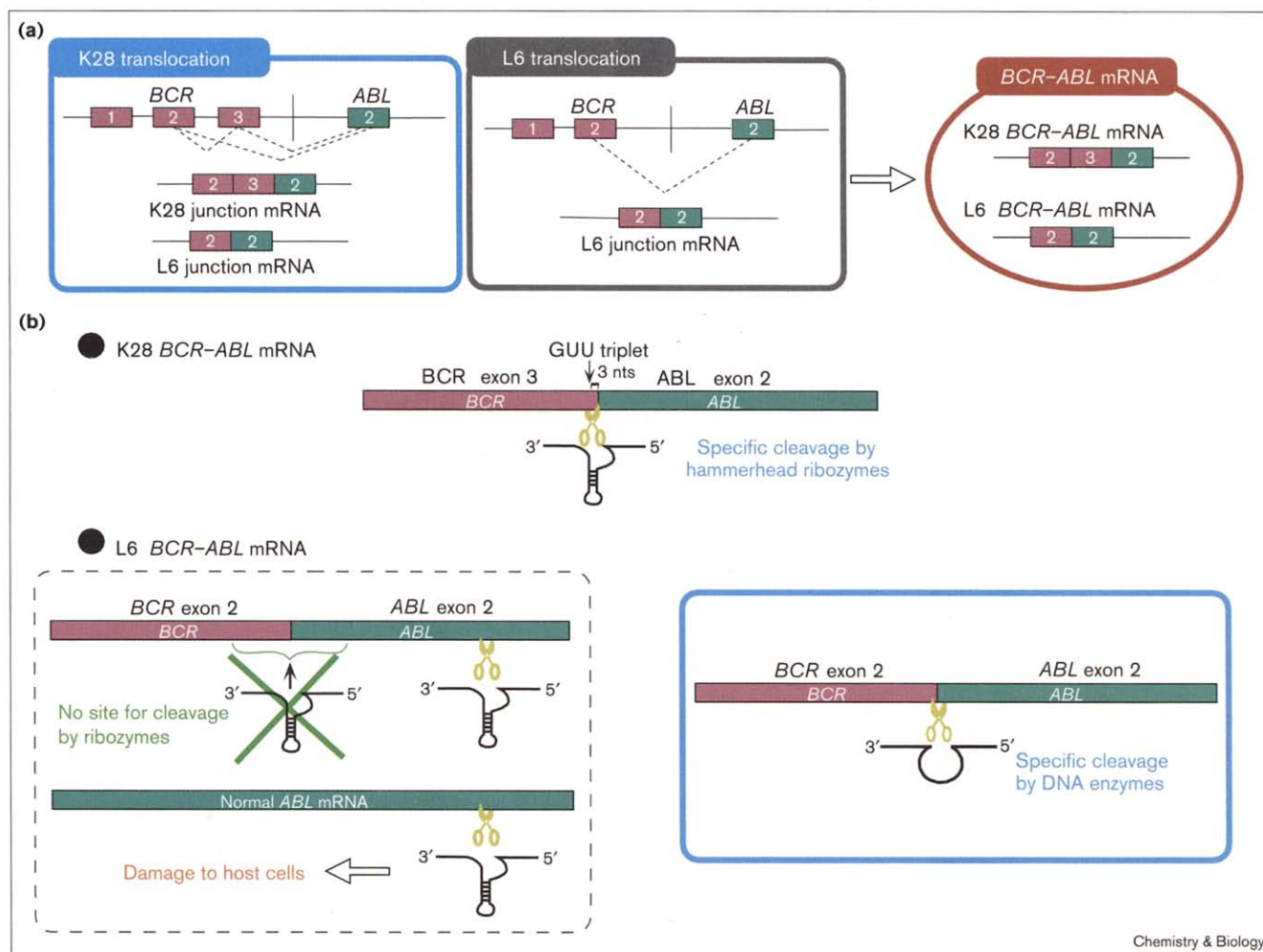
The Philadelphia chromosome is a cytogenetic abnormality that results from reciprocal chromosomal translocations t(9; 22) (q34; q11) and it is characteristic of cells from patients with chronic myelogenous leukemia (CML) [1]. The translocations can be subdivided into two types: K28 and L6 translocations. Both translocations result in the formation of the *BCR-ABL* fusion gene, which encodes two types of mRNA: b3a2 mRNA (consisting of *BCR* exon 3 and *ABL* exon 2) and b2a2 mRNA (consisting of *BCR* exon 2 and *ABL* exon 2; Figure 1a) [2-7]. Both these mRNAs are translated into a protein of 210 kDa (p210^{*BCR-ABL*}), which is unique to malignant CML cells [8].

In designing ribozymes and antisense DNA that can disrupt expression of a chimeric RNA specifically, it is necessary to target the junction sequence. Otherwise, expression of normal mRNAs that share part of the sequence of the chimeric RNA will also be affected, with resulting damage to host cells. In the case of b3a2 mRNA, a site potentially susceptible to cleavage by the hammerhead ribozyme is located three nucleotides upstream from the junction (Figure 1b). A conventional hammerhead ribozyme might therefore be expected to cleave specifically the abnormal b3a2 mRNA generated from K28 translocations. Indeed, several examples of such cleavage have been reported [9-16]. In contrast, in the case of b2a2 mRNA, which

results from L6 translocations, as well as in the case of some K28 translocations (Figure 1a), there are no hammerhead ribozyme target sequences within two or three nucleotides of the *BCR-ABL* junction. When the cleavage site is located further from the junction, nonspecific cleavage of normal mRNAs by hammerhead ribozymes cannot be avoided (Figure 1b). Previous attempts at engineering specific cleavage by the hammerhead ribozyme have involved a combination of a long antisense arm, which recognizes the junction, and a ribozyme sequence [17,18]. Hammerhead ribozymes can cleave RNAs even if one of the binding arms is only two or three nucleotides long [19,20]. We note that ribozymes other than the hammerhead, such as the hairpin, might cleave the *BCR-ABL* junction of b2a2 mRNA specifically and might, therefore, be worth investigating further. In the case of antisense DNAs, oligonucleotides of a certain length are necessary for stable binding of the antisense molecule to its target, resulting in decreases in specificity [21-27]. The utility of such antisense-type ribozymes (and antisense DNAs) remains questionable because of their low specificity [21-29].

In a recent study, Santoro and Joyce [30] successfully selected DNA enzymes that were able to cleave RNA molecules with any sequence by use of a selection procedure *in vitro*. These DNA enzymes are rather similar to conventional hammerhead ribozymes because they consist

Figure 1



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BCR-ABL translocations and fusion mRNAs. (a) The two types of chromosomal translocations (K28 and L6) that are associated with chronic myelogenous leukemia (CML) and the corresponding fusion mRNAs are depicted. Red boxes represent BCR exons and green boxes represent ABL exon 2. Dotted lines connecting BCR and ABL exons indicate alternative splicing pathways. **(b)** Possible sites of

cleavage by ribozymes and DNA enzymes are indicated by scissors. In L6 b2a2 mRNA, which mainly results from L6 translocations (and some K28 translocations), there are no triplet sequences near the BCR-ABL junction that are potentially susceptible to cleavage by hammerhead ribozymes. In contrast, DNA enzymes can cleave L6 b2a2 mRNA because of their high flexibility in the choice of cleavage site.

of a 15 deoxyribonucleotide catalytic domain and, in addition, metal ions, such as Mg^{2+} , are necessary for catalytic activity ([31,32]; Zhou and K.T., unpublished observations), as is the case for hammerhead ribozymes, which are recognized as metalloenzymes [33–44]. The catalytic domain is flanked by two substrate-recognition domains of seven or eight deoxyribonucleotides each, and the RNA substrate is bound through Watson–Crick base pairing (Figure 2a). These DNA enzymes can be divided into two types. Type I DNA enzymes can cleave an RNA sequence at a phosphodiester bond that is located between an adenine and a guanine residue. The catalytic domain consists of a four-nucleotide loop adjacent to the cleavage site and a stem-loop region that resembles the stem-loop

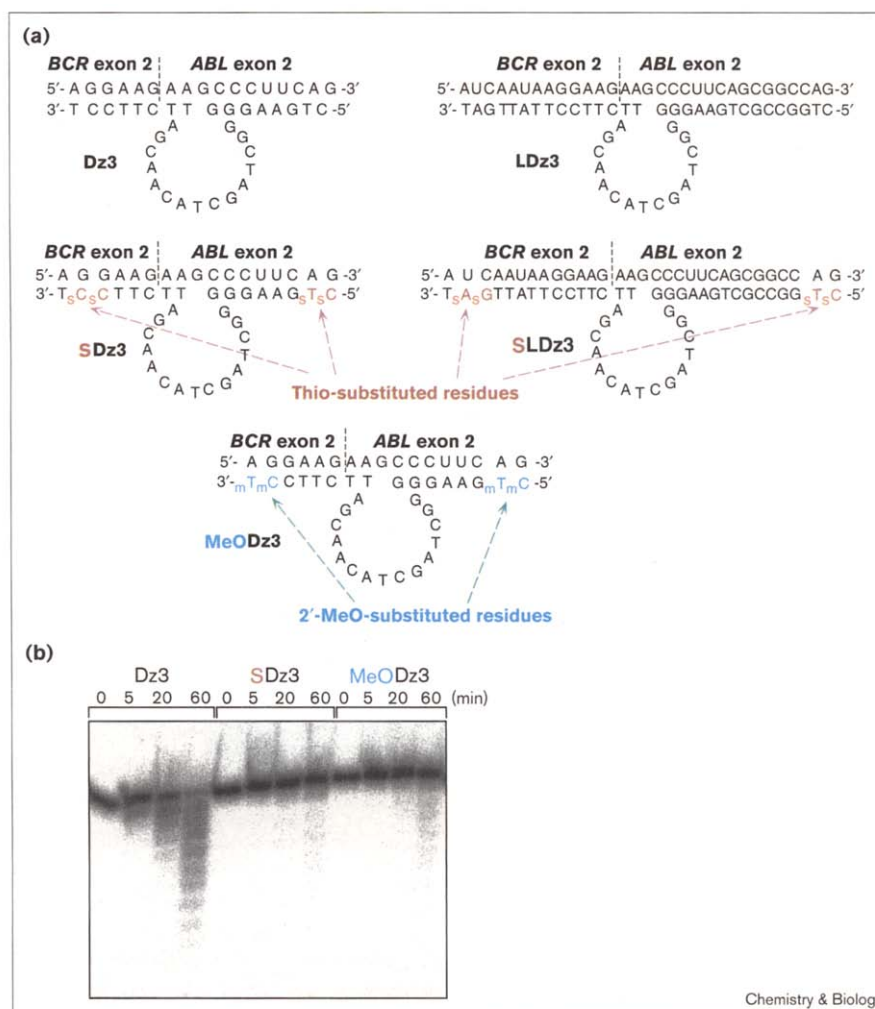
region II of the hammerhead ribozyme. The stem-loop region in the DNA enzyme is essential for catalysis, however [30]. Type II DNA enzymes cleave an RNA sequence at a phosphodiester bond between a purine and pyrimidine residue. In this case, the catalytic domain consists of 15 nucleotides (Figure 2). Such DNA enzymes can be expected to cleave almost any target RNA substrate specifically. With respect to substrate specificity, these DNA enzymes might have other advantages, when compared with hammerhead ribozymes. In general, DNA–RNA duplexes are less stable than the corresponding RNA–RNA duplexes. In the case of a DNA enzyme, therefore, mismatches in binding arms that might be generated during binding with a nontarget mRNA, such as

Figure 2

DNA enzymes used in this study.

(a) Nucleotide sequences of DNA enzymes targeted to the L6 *BCR-ABL* (b2a2) mRNA substrate. Red and blue letters indicate phosphorothioate linkages and 2'-O-Me-substituted residues, respectively.

(b) Stabilities of DNA enzymes in human serum. Fully ^{32}P -labeled DNA enzyme (20 kcpm) was incubated in 90% human serum at 37°C for the indicated period. Degraded products were separated by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel and were detected by a Bio-Image Analyzer (BAS2000; Fuji Film, Tokyo).



normal *ABL* mRNA, would inhibit cleavage activity to a greater extent than in the case of a ribozyme.

We are interested in the specific cleavage of b2a2 mRNA. There are several sites of potential cleavage by the DNA enzymes within three nucleotides of the *BCR-ABL* junction in this mRNA. We demonstrated previously that the DNA enzymes specifically cleave *BCR-ABL* chimeric L6 (b2a2) mRNA *in vitro* [28]. We found that our DNA enzyme, designated Dz3 (which originated from a type II DNA enzyme), was the most efficient of all those that we tested [28]. The usefulness of Dz3 *in vivo* remained to be examined, however. In this study, we investigated the activity and specificity of Dz3 in cultured cells. To cleave a chimeric mRNA such as *BCR-ABL* mRNA, specific recognition of abnormal mRNA and the absence of any effect on normal mRNA are essential. Accordingly, we also investigated the effects of introducing modified oligonucleotides into Dz3 on specificity and cleavage activity in mammalian

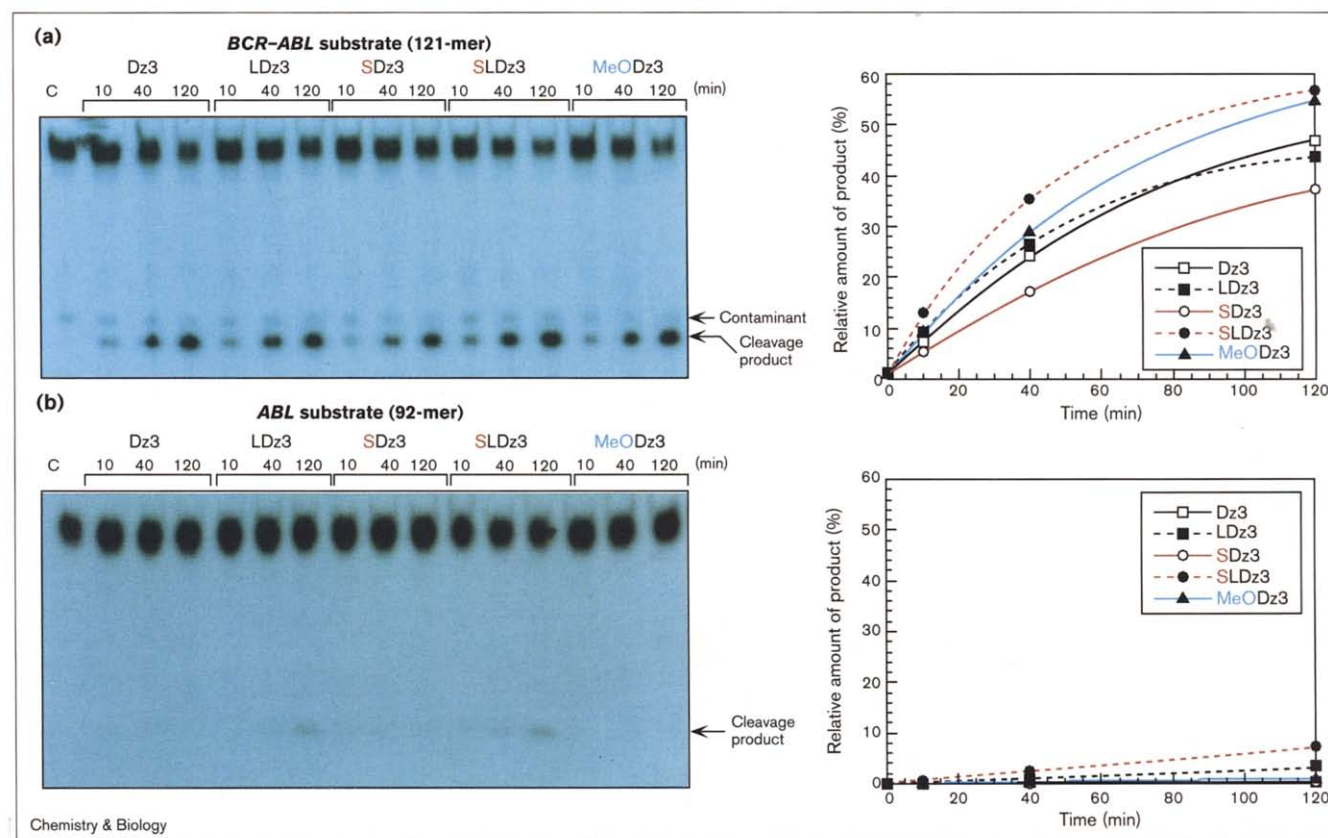
cells using reporter constructs that included a luciferase gene. In addition, we monitored cleavage of an endogenous target in leukemic BV173 cells.

Results and discussion

Design of DNA enzymes and stability of DNA enzymes in human serum

The design of the DNA enzymes and the choice of the target site for the specific cleavage of L6 *BCR-ABL* (b2a2) mRNA were based on results published previously [28]. In a previous study we found that the most effective DNA enzyme *in vitro* was Dz3, 31 nucleotides (nt) in length, that cleaves between guanine and cytosine residues on the 3' side of the junction of *BCR* exon 2 and *ABL* exon 2 in L6 *BCR-ABL* mRNA. Moreover, Dz3 did not cleave normal *ABL* mRNA *in vitro*. To test the possibility of using such a DNA enzyme in mammalian cells, we examined several variants of Dz3. Dz3 has a catalytic domain of 15 nt in length that is flanked by two 8-nt substrate-binding

Figure 3



Cleavage activities and specificities of DNA enzymes *in vitro*. (a) Relative extents of DNA-enzyme-mediated cleavage of the BCR-ABL substrate mRNA (121-mer). (b) Specificity of DNA-enzyme-mediated cleavage of ABL substrate mRNA (92-mer). Each DNA

enzyme (1 μ M) and 5'- 32 P-labeled substrate (2 nM) were incubated at 37°C for the indicated times in a solution that contained 50 mM Tris-HCl (pH 8.0) and 25 mM MgCl₂. Each reaction mixture was then subjected to electrophoresis on a 5% polyacrylamide/7 M urea gel.

arms. In general, for efficient catalysis *in vivo*, the length of the antisense arms that recognize the target sequence and the introduction of modified oligonucleotides to enhance resistance to nucleases *in vivo* are both important. We therefore prepared longer-armed Dz3 (LDz3) and several modified Dz3 forms, as shown in Figure 2a. LDz3 has binding arms of 15 nt each. SDz3 and SLDz3 corresponded to Dz3 and LDz3 but have two phosphorothioate substitutions at both their 5' and 3' ends (for a total of four modifications). MeODz3 corresponds to Dz3 but has two 2'-O-methyl-substituted residues at both the 5' and the 3' ends (for a total of four modifications).

To confirm the nuclease resistance of the modified DNA enzymes, we analyzed the stability of Dz3, SDz3 and MeODz3 in human serum. Fully 32 P-labeled oligonucleotides were incubated in 90% human serum (collected as described in [45]) for 5, 20 and 60 min at 37°C and the products were analyzed by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel. As can be seen in Figure 2b, significant portions of the Dz3 were degraded

after 60 min. In contrast, the modified SDz3 and MeODz3 remained intact, even after a 60 min incubation. It is clear, therefore, that introducing two phosphorothioate linkages or two 2'-O-methyl-substituted residues at both the 5' and 3' ends (for a total of four modifications) significantly enhanced resistance to nucleases.

Cleavage activities and specificities of DNA enzymes for the chimeric BCR-ABL L6 (b2a2) mRNA substrate

Before examining the DNA enzymes in mammalian cells we investigated the effects of the modifications described above on cleavage activity and specificity against the chimeric BCR-ABL (b2a2) substrate and the normal ABL substrate (which are 121 and 92 nt long, respectively). The relative cleavage activities and the specificities of each DNA enzyme for the chimeric BCR-ABL substrate (121-mer) and the normal ABL substrate (92-mer) are shown in Figure 3. Reactions were performed in 50 mM Tris-HCl (pH 8.0) and 25 mM MgCl₂ under enzyme-saturating (single-turnover) conditions at 37°C. Each of the DNA enzymes cleaved the BCR-ABL substrate at the

Table 1**Kinetic parameters of cleavage of the *BCR-ABL* substrate mRNA (121-mer).**

DNA enzyme	k_{cat} ($\times 10^{-2} \text{ min}^{-1}$)	K_M (μM)	k_{cat}/K_M ($\mu\text{M}^{-1} \text{ min}^{-1}$)
Dz3	0.82	0.11	0.075
LDz3	1.3	0.012	1.1
SDz3	0.52	0.10	0.052
SLDz3	1.7	0.010	1.7
MeODz3	1.0	0.10	0.10

Rate constants were measured in 50 mM Tris-HCl (pH 8.0) and 25 mM MgCl_2 under enzyme-saturating (single-turnover) conditions at 37°C. Rate constants are averages of results from two sets of experiments.

anticipated site, and neither the thio substitutions nor the 2'-MeO residues significantly inhibited the cleavage activity (Dz3, lanes 2–4; SDz3, lanes 8–10; MeODz3, lanes 14–16; Figure 3a). These results were not surprising because the introduced modifications were sufficiently far from the catalytic domain, as had also been the case for modified synthetic hammerhead ribozymes [45–49].

Highly specific DNA enzymes should cleave only the chimeric *BCR-ABL* substrate. In contrast, DNA enzymes with reduced specificity would be expected to cleave not only the chimeric *BCR-ABL* substrate but also the normal *ABL* mRNA because one of the two recognition arms of our various DNA enzymes can hybridize with part of the normal *ABL* mRNA. As can be seen from Figure 3b, the modifications by themselves did not affect the specificity of the DNA enzymes and no products of cleavage of the normal mRNA were detected (Dz3, lanes 2–4; SDz3, lanes 8–10; MeODz3, lanes 14–16). In contrast, however, DNA enzymes that have longer binding arms (LDz3 and SLDz3) did cleave the normal *ABL* mRNA to a limited extent; this result indicates that these DNA enzymes with their relatively long antisense arms recognized not only the abnormal *BCR-ABL* mRNA but also the normal *ABL* mRNA as substrate. Nonspecific cleavage of normal *ABL* mRNA occurred when we used longer-armed DNA enzymes *in vitro*. This is because, in order to amplify the detection of the nonspecific cleavage, we used high concentrations of Mg^{2+} (25 mM MgCl_2) in the *in vitro* experiment. We should mention that the same longer-armed DNA enzymes acted with high specificity within mammalian cells, as discussed below.

In order to characterize in further detail the properties of the modified DNA enzymes and the longer-armed DNA enzymes, we determined kinetic parameters for the cleavage of the *BCR-ABL* 121-mer mRNA substrate under single-turnover conditions. The kinetic parameters (k_{cat} and K_M) are summarized in Table 1. Comparisons of kinetic parameters revealed the absence of any significant differences in k_{cat} and K_M values between the modified

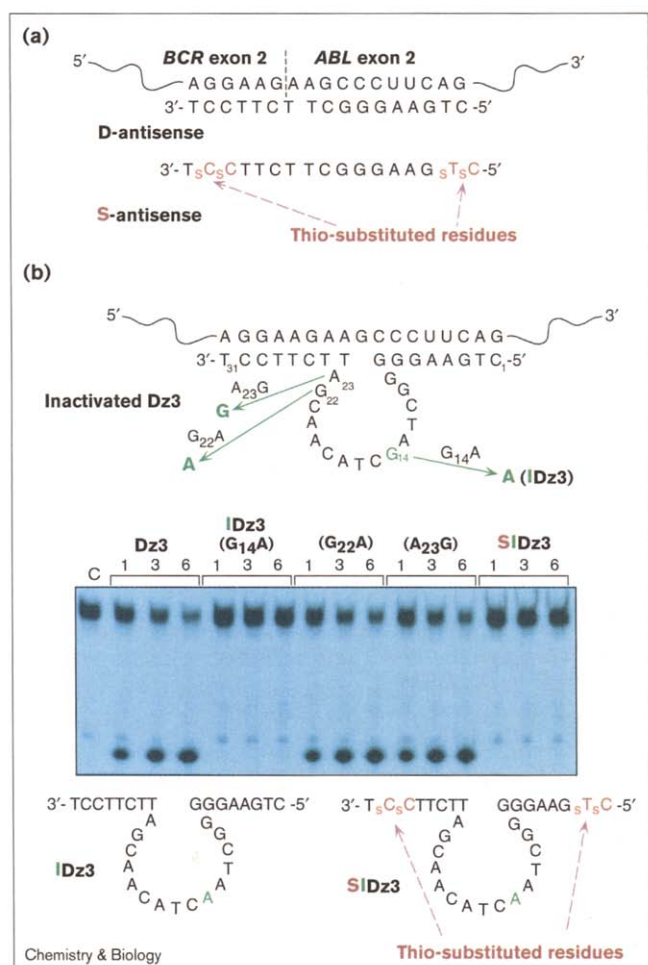
and the unmodified DNA enzymes (Table 1). In the case of the longer-armed DNA enzymes, which have approximately twice as many nucleotides in their binding arms as the parental DNA enzymes, we found that the longer the binding arms, the higher the k_{cat} values and the lower the K_M values (i.e. overall higher k_{cat}/K_M values; Table 1). In terms of k_{cat}/K_M , Dz3 was shown previously to be more powerful when a short synthetic oligonucleotide substrate of 21 nt was used [28]. It is generally accepted that long mRNAs, because of their higher-ordered structures, are cleaved less efficiently by ribozymes than corresponding short synthetic oligoribonucleotide substrates [50–52]. This was also the case for cleavage by DNA enzymes (data not shown).

Comparison of the intracellular activities and specificities of the unmodified and modified DNA enzymes using a reporter construct in cultured cells

Having confirmed the specificities and activities of our series of DNA enzyme *in vitro*, we next examined their effects in cultured cells using a reporter construct. In order to distinguish between the chemical-cleavage activities of our DNA enzymes and their antisense effects, we prepared both inactive DNA enzymes and antisense oligonucleotides as controls. As shown in Figure 4a, antisense DNA (D-antisense), which consisted entirely of deoxyribonucleotides, had a sequence complementary to the target sequence of Dz3. The modified antisense molecule, S-antisense, had two phosphorothioate substitutions at its 5' and 3' ends, as did SDz3. To identify an inactive DNA enzyme, we synthesized three variants of Dz3 ($G_{14} \rightarrow A$, $G_{22} \rightarrow A$ and $A_{23} \rightarrow G$ in Figure 4b) and examined their cleavage activities *in vitro*. $G_{14}A$ has an A residue instead of G_{14} in the parental Dz3. $G_{22}A$ and $A_{23}G$ have A and G residues instead of the G_{22} and A_{23} residues in the parental Dz3, respectively. Cleavage of the *BCR-ABL* substrate (121-mer) by these DNA enzyme variants is shown in Figure 4b. Because $G_{14}A$ had no cleavage activity, even when incubated with the substrate for 6 h, we used it as an inactive DNA enzyme in subsequent studies, renaming it IDz3 (Figure 4b). We also prepared thio-substituted IDz3, which we named SIDz3.

To evaluate the intracellular activities of the various oligonucleotides (Dz3, LDz3, IDz3, SDz3, SLDz3, SIDz3, MeODz3, D-antisense and S-antisense), we co-transfected HeLa cells with the oligonucleotides, together with a target-gene-expressing plasmid that encoded a chimeric target *BCR-ABL* (or *ABL*) sequence and a gene for luciferase, pB2A2-luc (or p*ABL*-luc) in combination with Lipofectin (Figure 5). The junction-expressing plasmid pB2A2-luc contained a 300-nt sequence that encompassed the *BCR-ABL* junction of L6 b2a2 mRNA. The plasmid p*ABL*-luc contained a 300-nt sequence that encompassed the junction between exon 1 and exon 2 of normal *ABL* mRNA. After co-transfection with the oligonucleotides

Figure 4



Nucleotide sequences of antisense oligonucleotides and an inactive DNA enzyme. (a) Antisense DNA (D-antisense) targeted to the b2a2 junction of *BCR-ABL* mRNA and its thio-modified derivative (S-antisense; thio-substituted residues are red). (b) Selection of an inactive DNA enzyme (IDz3). Three types of variant DNA enzymes with base substitutions at the indicated sites were examined with respect to cleavage of the *BCR-ABL* substrate (121-mer). $G_{14} \rightarrow A$ is inactive and was renamed IDz3. The thio-substituted residues of SIDz3 are indicated.

and transient expression of target genes for 12 h, we estimated the intracellular activity of each oligonucleotide by measuring the luciferase activity in individual cell lysates.

The inhibitory effects were tested at two different oligonucleotide concentrations and the results of one set of the experiments are summarized in Figure 6. The luciferase activity recorded when we used each target-gene-expressing plasmid (pB2A2-luc or pABL-luc) by itself was taken as 100%. When 0.3 μ M oligonucleotide was used (Figure 6), to our surprise, the unmodified parental Dz3 suppressed the expression of the *BCR-ABL*-luciferase gene in mammalian

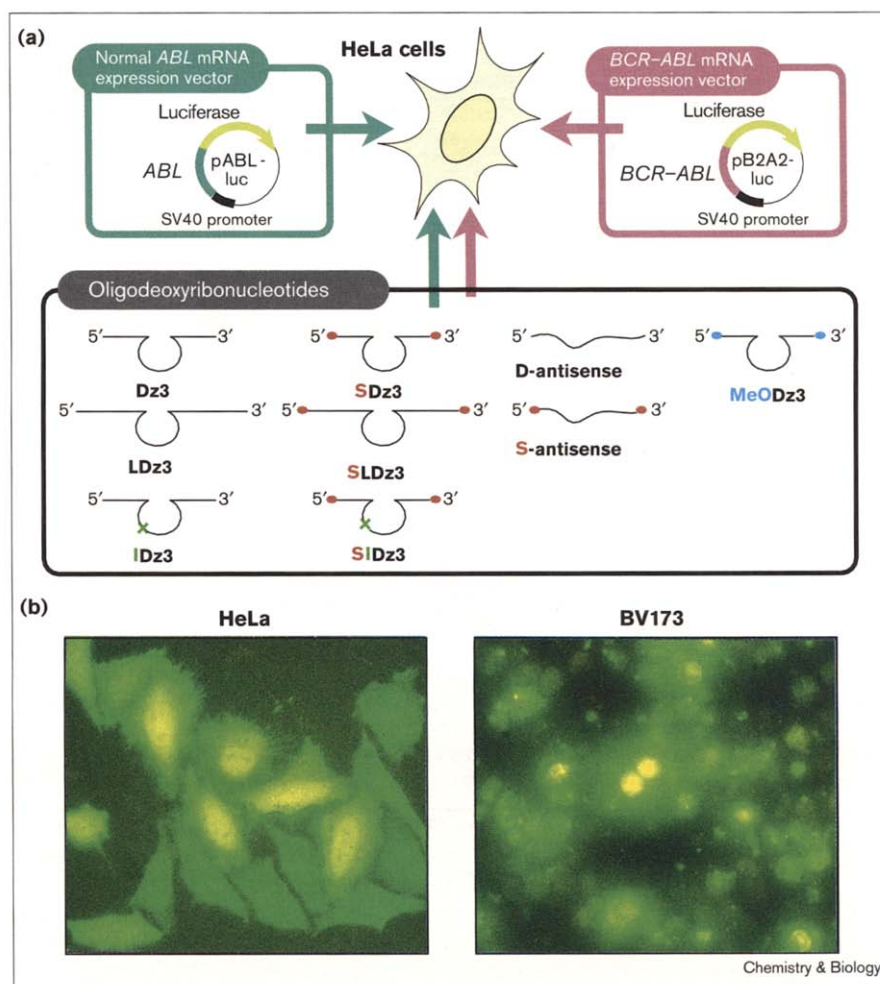
cells both specifically and with extremely high efficacy (~99% inhibition). Moreover, it had no inhibitory effect on the expression of the normal *ABL*-luciferase gene (no side effects), as expected from our analysis *in vitro*. LDz3, which had slight nonspecific cleavage activity against the normal *ABL* substrate *in vitro*, exhibited not only high efficacy but also high specificity in suppression of the *BCR-ABL*-luciferase gene in cultured cells. Although both enzymes were very active, LDz3 seemed to be slightly more active than Dz3.

In contrast to Dz3, D-antisense inhibited the expression of both the *BCR-ABL*-luciferase gene and the *ABL*-luciferase gene. Many attempts at using antisense oligonucleotides to suppress specifically the expression of *BCR-ABL* fusion mRNA have been reported [21–27]. Despite the initial optimism, recent studies have failed to demonstrate that any appropriate antisense oligonucleotide can specifically suppress the expression of *BCR-ABL* fusion mRNA. It has also been suggested that nonspecific inhibition by such antisense oligonucleotides is the result of nonantisense effects [21–27]. In agreement with such recent reports, our antisense DNAs failed to suppress specifically the reporter constructs in HeLa cells. DNA enzymes include antisense sequences that allow them to recognize their target mRNA, but these sequences are interrupted by the catalytic loop and, as a result, lower affinity and higher specificity for the substrate are expected. Indeed, as shown in Figure 6, the inactive DNA enzyme IDz3 did not have any inhibitory effect on the expression of the *BCR-ABL*-luciferase gene or the *ABL*-luciferase gene. This result also demonstrates that the inhibitory effects of the DNA enzymes did not originate from antisense effects but from the specific chemical-cleavage activity of the DNA enzymes themselves. Moreover, although the inactive DNA enzymes could potentially promote RNase-H-mediated cleavage of the substrate as observed in experiments *in vitro* (Figure 7; the level of RNase H was adjusted such that both DNA-enzyme- and RNase-H-mediated cleavage products were discernible on the gel), the results shown in Figure 6 clearly demonstrate that the inhibitory effects of DNA enzymes did not originate from the RNase H activity in cells. Apparently, the specific chemical-cleavage activity of the DNA enzymes was significantly higher than the RNase-H-mediated cleavage activity *in vivo*, at least under the conditions of our investigations.

To use such DNA enzymes *in vivo*, protection from intracellular nucleases is obviously essential. Various kinds of nuclease-resistant oligonucleotide have therefore been developed [21–27,45–49,53,54]. The most popular modification remains the introduction of phosphorothioate linkages. Previous attempts at using antisense oligonucleotides with thio-substituted residues to treat CML suggested that the nonsequence-specific inhibition was due to a

Figure 5

In vivo assay system. (a) Schematic representation of the assay for determining DNA enzyme activity in HeLa cells. (b) Photomicrographs of HeLa cells and BV173 leukemic hematopoietic cells transfected with the fluorescently labeled DNA enzyme. Cells were transfected with fluorescein end-labeled DNA enzyme (Dz3) at 0.3 μ M concentrations as described in the Materials and methods section. Samples were examined using a fluorescent microscope 12 hours after transfection (Nikon, Tokyo).

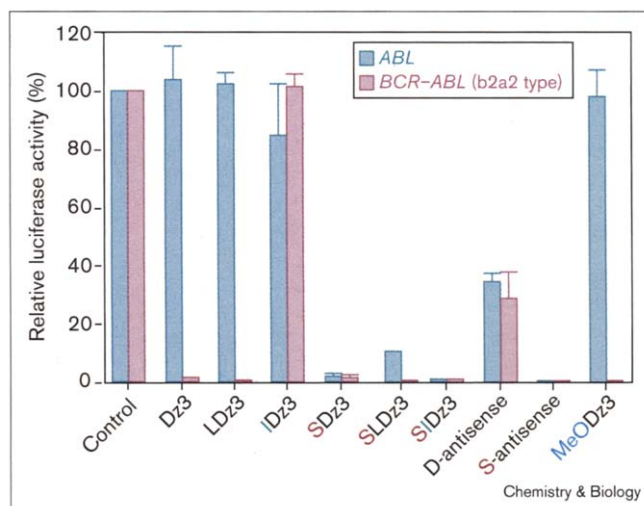


nonantisense mechanism [21–27]. In order to investigate the effects of thio substitutions, we designed four types of partially thio-substituted oligonucleotides, namely, SDz3, SLDz3, SIDz3 and S-antisense, as described above, and we examined their activities in mammalian cells (Figure 6). In most previous studies, almost all the linkages of antisense oligonucleotides have been thio substituted. In the present study, however, we introduced only four thio substitutions into each oligonucleotide, hoping to minimize nonsequence-specific effects. Nevertheless, SDz3, SLDz3, SIDz3 and S-Antisense all had strong inhibitory effects not only on the expression of the *BCR-ABL*-luciferase gene but also on the expression of the *ABL*-luciferase gene in HeLa cells. In particular, despite the fact that Dz3 and LDz3 specifically inhibited the expression of the *BCR-ABL*-luciferase gene, no similar specificity was observed when the respective thio-substituted counterparts were tested. Our assay system, as shown schematically in Figure 5a, is clearly useful for estimating the efficacy and specificity of functional

oligonucleotides in mammalian cells. Moreover, our present results highlight the danger of introducing thio linkages even in limited numbers.

The inhibitory effects of the thio-substituted oligonucleotides were also tested at higher concentrations (10 μ M, data not shown). In this assay, in contrast to the experiments summarized in Figure 6 (in which the oligonucleotides and the target-gene-expressing plasmid were simultaneously added to cells), because of the higher concentrations of oligonucleotides used, the target-gene-expressing plasmids were introduced first to cells with Lipofectin and then, 2 h after incubation, the oligonucleotides were introduced to the cells again using Lipofectin. As a result, when the oligonucleotides were introduced, the cells had already produced some luciferase, reflecting the higher background level of luciferase activity. Here again, in agreement with the results shown in Figure 6, the DNA enzymes, with the exception of the thio-substituted enzymes, suppressed the expression of

Figure 6



Intracellular activities and specificities of DNA enzymes in HeLa cells. The effects of DNA enzymes on the chimeric *BCR-ABL*-luciferase gene and the *ABL*-luciferase gene (red and green, respectively) were detected with 0.3 μ M DNA enzyme. HeLa cells were co-transfected with each oligonucleotide together with the target gene-expressing plasmid in combination with Lipofectin. Luciferase activity was normalized by reference to the efficiency of transfection, which was determined by monitoring the activity of the product of a co-transfected gene for β -galactosidase (see the Materials and methods section). These data are averages of results from two sets of experiments.

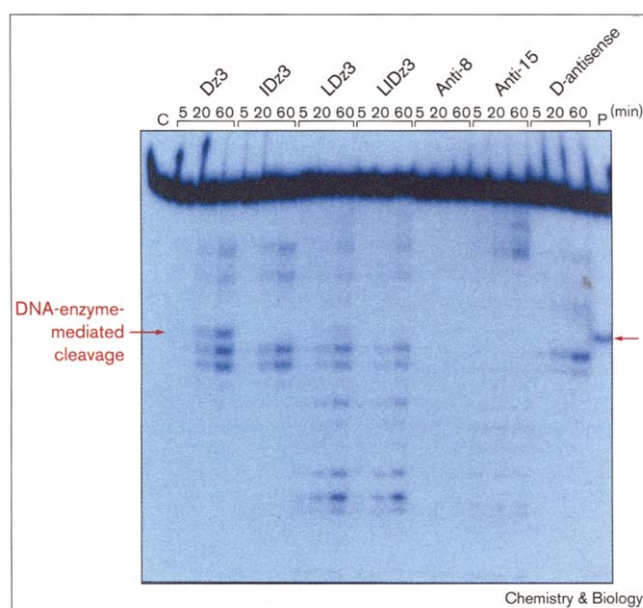
the *BCR-ABL*-luciferase gene in mammalian cells both specifically and with high efficacy (data not shown).

In contrast to the thio-substituted DNA enzymes, the DNA enzyme with two 2'-methoxy-substituted residues at each end (MeODz3) specifically and effectively suppressed expression of the *BCR-ABL*-luciferase gene in cultured cells. This result suggests that such a DNA enzyme could potentially be used *in vivo* and also suggests that 2' methoxy groups are suitable for modifying such enzymes. Introducing phosphorothioate linkages, which resulted in nonspecific damage not only to the abnormal gene but also to the normal gene, seems to be less suitable. In designing oligonucleotides with the potential to disrupt chimeric RNAs, it is necessary to avoid nonspecific effects by the introduced modifications on normal genes.

Efficacies and specificities of the unmodified and modified DNA enzymes against an endogenous *BCR-ABL* cellular target in BV173 cells

Because our DNA enzymes acted with high efficacy and specificity against a reporter-gene construct in HeLa cells, we decided to examine the activity of the DNA enzymes against an endogenous *BCR-ABL* (L6 b2a2 mRNA) cellular target. Because BV173 cells, which were originally derived from a CML patient, are Philadelphia chromosome-positive, they express the endogenous gene target

Figure 7



RNase H-mediated cleavage of the RNA substrate (S31). After hybridization of S31 with the respective DNA enzyme or antisense DNA, reactions with RNase H were initiated under conditions where both DNA-enzyme- and RNase H-mediated cleavage could occur. The substrate and products were separated by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel and were detected by autoradiography. C, labeled S31 (control); P, labeled S31 treated with Dz3 in the absence of RNase H. Red arrows indicate the cleavage products mediated by DNA enzymes.

[21–27,55]. In BV173 cells, disrupting expression of the *BCR-ABL* gene induces apoptosis [21–27]. We compared the efficacies of Dz3, SDz3 and MeODz3, as representative DNA enzymes, together with the inactive control (IDz3), by examining the viability of BV173 cells that had been transfected with each DNA enzyme. In addition to BV173 cells, we used H9 cells, which are derived from human T cells, and express normal *ABL* mRNA, as a control. We confirmed, using a fluorescently labeled oligonucleotide (Dz3 with 6-carboxyfluorescein at its 5' end) and Lipofectin, that all these cells could be transfected with oligonucleotides (Figure 5b).

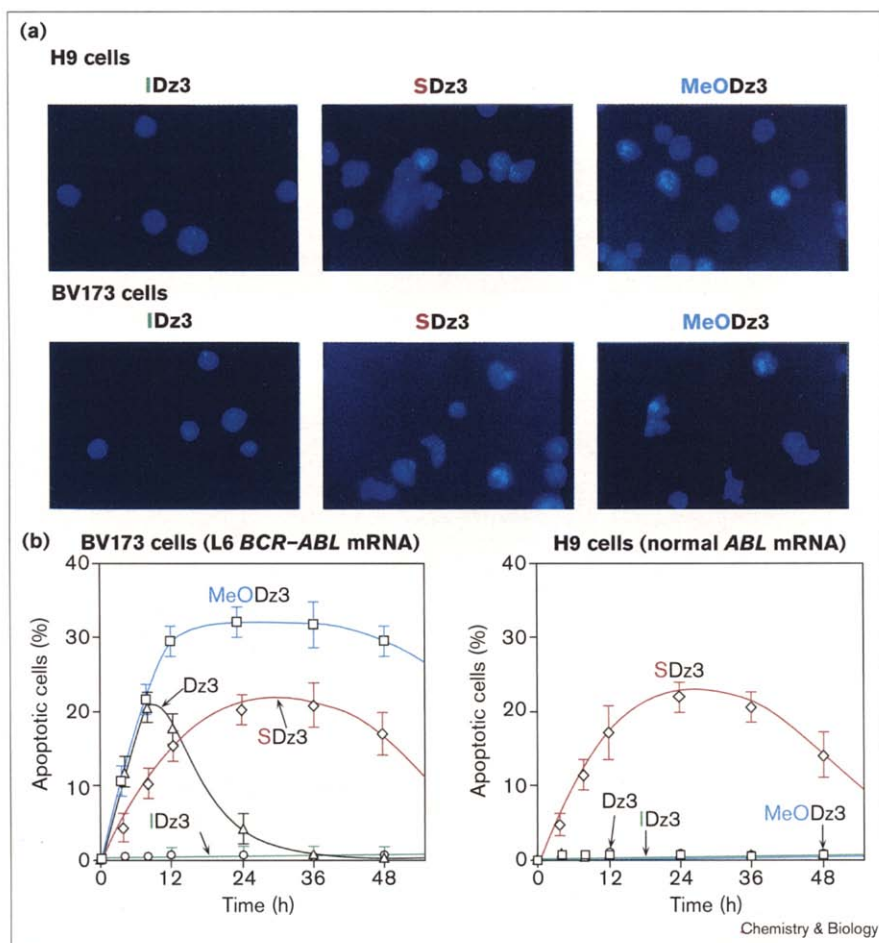
We first examined MeODz3 as the best representative example of our DNA enzymes. We expected that it would exhibit high efficacy and specificity; in addition to nuclease resistance, we examined its effects on H9 and BV173 cell morphology. Upon introduction of MeODz3, an examination of BV173 cells by light microscopy, after staining with the DNA-binding fluorochrome Hoechst 33342, revealed typical apoptotic morphology, which included condensed chromatin, fragmented nuclei and shrunken cell profiles (Figure 8a, bottom right). It was clear that MeODz3 caused apoptotic cell death specifically in BV173 cells and had no similar effect on normal H9 cells (Figure 8a, top right). As

Figure 8

Efficiency of DNA-enzyme-mediated cleavage of an endogenous *BCR-ABL* mRNA target.

(a) Morphology of H9 and BV173 cells treated separately with IDz3, SDz3 or MeODz3. After 12 h from transfection of each DNA enzyme, cells were stained with 10 μ g/ml Hoechst 33342 (DNA-binding fluorochrome) for 15 min for studies of nuclear morphology. After washing and mounting in 90% glycerol/20 mM Tris (pH 8.0)/0.1% *N*-propyl gallate, slides were examined under a fluorescent microscope (Nikon, Tokyo). Not only BV173 cells introduced with SDz3 and MeODz3, but also H9 cells introduced with SDz3 exhibited typical apoptotic morphology.

(b) Viability of BV173 and H9 cells treated with DNA enzymes. Cell viability was determined using the trypan blue exclusion test. These data are averages of results from two sets of experiments.



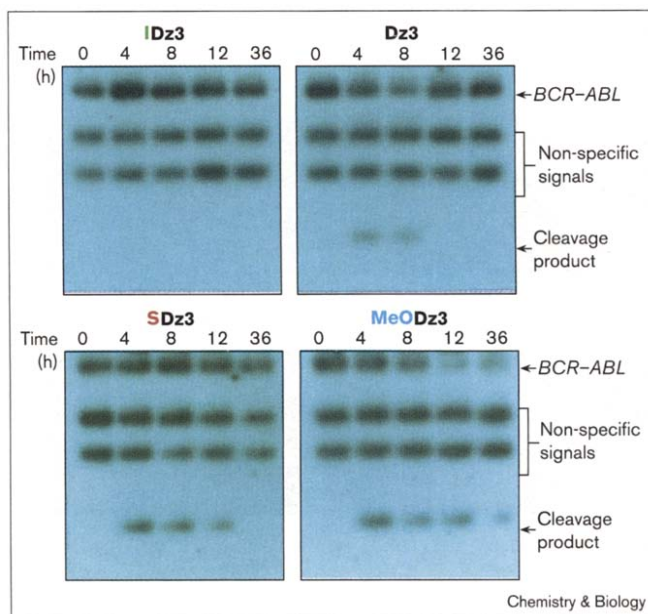
expected, SDz3 caused apoptotic cell death not only in BV173 cells but also in H9 cells. Because IDz3 did not change the morphology of either cells line, the apoptotic cell death caused by MeODz3 must have originated from its cleavage activity in BV173 cells.

In order to analyze quantitatively the efficacies and specificities of three of our DNA enzymes, Dz3, SDz3 and MeODz3, in BV173 and H9 cells, we examined the ability of cells to exclude trypan blue dye after transfection with each DNA enzyme in combination with Lipofectin. As can be seen in Figure 8b, for the first 8 h, each DNA enzyme exhibited high but dissimilar activity in BV173 cells. Unmodified, nuclease-sensitive Dz3 started to lose activity about 10 h after transfection, and neither Dz3 nor MeODz3 caused the death of any H9 cells, which expressed normal *ABL* mRNA (Figure 8b), demonstrating the expected high specificity for targeting the chimeric *BCR-ABL* gene (i.e. the absence of side effects in normal cells). In contrast, SDz3 induced the death of both BV173 and H9 cells, consistent with the result that

thio-substituted oligonucleotides inhibited the expression of both *BCR-ABL* and normal *ABL* genes in the reporter constructs (Figure 6). In most studies, antisense oligonucleotides are added several times periodically to cells; we added each DNA enzyme to cells only once in the present study. The decrease in the relative number of apoptotic cells 8 h after addition of Dz3 and 36 h after the addition of SDz3 is most probably due to degradation of the DNA enzymes by nucleases. MeODz3 had the longest-lasting continuous activity against the endogenous target gene, a result that underlines, yet again, the appropriate nature of the 2' methoxy modification.

Evidence against the cleavage of RNAs during their isolation procedures *in vitro*

Because the DNA enzymes were expected to overcome the *BCR-ABL*-mediated inhibition of apoptosis by cleaving *BCR-ABL* mRNA, we tried to detect the anticipated cleavage products directly using Northern blotting analysis (Figure 9). It is possible that the target mRNAs were cleaved by the DNA enzymes during the RNA extraction

Figure 9

Direct detection of the products of cleavage, within BV173 cells, of L6 *BCR-ABL* mRNA by Northern blotting analysis. As long as a monophasic solution of phenol is used for the extraction of total RNAs [29,56–58], no cleavage occurs during RNA isolation. See text for details.

process. In order to exclude this possibility, a parallel, control experiment was set up. Total RNA from BV173 cells that did not contain DNA enzyme was extracted with the extraction buffer (a monophasic solution of phenol; see the Materials and methods section) that contained the active DNA enzyme MeODz3. Using Northern blotting analysis, we were only able to detect intact *BCR-ABL* mRNA; no cleavage products were detected (data not shown). This finding is in agreement with our previous result with ribozymes [29]. In a control experiment, cells (BaF3/p210^{BCR-ABL} cells) expressing only the target RNA (substrate) were mixed, just before the RNA-isolation procedure, with cells (transformed BaF3 cells) that expressed only the ribozyme, and the total RNA was isolated from the mixture [29]. Northern blotting didn't detect any cleavage products, a clear demonstration that the cleavage had occurred within the cells but not during the RNA isolation procedure *in vitro*. These results led us to the conclusion that the apoptosis observed was a result of cleavage of *BCR-ABL* mRNA by the ribozyme, with resultant depletion of p210^{BCR-ABL} protein in the hematopoietic cells (the p210^{BCR-ABL} protein is a cytoplasmic, membrane-associated protein that has a constitutively high level of tyrosine kinase activity that prolongs the survival of hematopoietic cells by inhibiting apoptosis).

The above-mentioned two sets of control experiments clearly demonstrate that, as long as a monophasic solution

of phenol is used for RNA extraction [29,56–58], the cleavage products we detect using Northern blotting analysis do not originate from cleavage during the RNA isolation procedure but from cleavage within the cells.

Direct evidence for cleavage of L6 *BCR-ABL* mRNA by the DNA enzymes

Total RNA from DNA enzyme-treated BV173 cells were extracted 0, 4, 8, 12 and 36 h after the addition of each DNA enzyme. The lengths of the L6 *BCR-ABL* target mRNA (about 8.5 kilobases; top band Figure 9) and the cleavage product (about 5.3 kilobases; bottom band) were exactly as anticipated. The two middle bands are non-specific and thus could be washed off by more extensive and rigorous washing at a higher temperature when a longer probe was used (data not shown; a shorter probe was used in Figure 9). Because the bands were sharper in the Northern blotting data shown in Figure 9 than those obtained with the longer probe, we display those Northern blotting data with nonspecific bands in Figure 9. No reduction in the level of expressed L6 *BCR-ABL* mRNA and no cleaved product was detected in the presence of the inactive DNA enzyme IDz3 (Figure 9a). In contrast, in all cases of treatment with active DNA enzymes, we detected cleaved fragments (Figure 9b–d). Although the Dz3-cleavage product was detected in total RNA extracted 4 and 8 h after the addition of Dz3, the cleaved product disappeared and the amount of intact L6 *BCR-ABL* mRNA returned to the basal level after prolonged incubation (consistent with the results in Figure 8b). In the case of SDz3, the cleaved product was also detected, indicating that SDz3 retained cleavage activity in cells in spite of the nonsequence-specific effects due to phosphorothioate linkages. Most importantly, it was clear that the amount of cleaved product was larger and the product was continuously detectable for a longer time in cells treated with MeODz3 (Figure 9d) than in cells treated with Dz3 or SDz3.

Detection of the cleaved fragment proved that the DNA enzymes were catalytically active in cultured cells. Moreover, we confirmed that the apoptosis of cells, as shown in Figure 8, with the exception of cells treated with SDz3, originated from the cleavage of *BCR-ABL* mRNA by a DNA enzyme, with resultant depletion of the p210^{BCR-ABL} protein in BV173 cells [29].

Significance

Chronic myelogenous leukemia (CML) cells are cytogenetically characterized by the Philadelphia chromosome, which results from translocations on chromosome 22. The translocations create *BCR-ABL* fusion genes, which encode two abnormal mRNAs (b3a2 and b2a2). Because both the abnormal p210^{BCR-ABL} chimeric protein and the wild-type p145 c-*ABL* protein are negative regulators of apoptosis [59–66], antisense molecules with low

specificity can induce apoptosis in CML cells by inhibiting expression of normal *ABL* mRNA (p145 c-*ABL*) in addition to blocking the *BCR-ABL* (p210^{BCR-ABL}) pathway. Previously designed antisense oligonucleotides that targeted the b3a2 or b2a2 mRNA had inhibitory effects on the proliferation of leukemic cells that expressed both b3a2 and b2a2 mRNAs, which clearly demonstrates the nonspecificity of these oligonucleotides. Moreover, inhibition by a nonantisense mechanism was also recognized [21–27].

In this study, we demonstrated that b2a2 *BCR-ABL* mRNA expression can be specifically inhibited by DNA enzymes. We examined expression of both reporter-gene constructs and an endogenous cellular target. The DNA enzymes exhibited high efficacy, and we confirmed, using Northern blotting analysis, that the DNA enzymes had cleavage activity in cultured cells and, moreover, that the apoptosis caused by the DNA enzymes in leukemic cells originated from the cleavage of the target *BCR-ABL* mRNA. Among the limited modifications that we introduced, 2'-OMe-substitution appeared to be the most effective, because introduction of two such modifications at each end of our DNA enzyme (MeODz3) resulted in significant resistance to nucleases without any loss of specificity. In contrast, using a thio-substituted version of the same DNA enzyme (SDz3) resulted in complete loss of specificity despite an increase in nuclease resistance.

In developing nucleic-acid drugs for treating CML, in particular in the case of the L6 translocations on which we focused our attention in this study, conventional ribozymes (with exception of a new motif described in [29,56,67]) might not be the best choice because of the lack of a suitable cleavage site in the target mRNA [28]. Similarly, because of a lack of substrate specificity against the chimeric target, antisense oligonucleotides might also not be appropriate. In contrast, the DNA enzymes used here exhibit high specificity for the chimeric target. Because DNA enzymes are easier to synthesize, easier to handle and more stable *in vivo* than ribozymes, appropriately modified DNA enzymes might turn out to be powerful catalytic nucleic-acid drugs.

Materials and methods

Synthesis of antisense DNAs and DNA enzymes

DNA enzymes and antisense DNAs were synthesized chemically on a DNA synthesizer (model 394; PE Applied Biosystems, Foster City, CA). Reagents for DNA synthesis were purchased from Glen Research (Sterling, VA). Oligonucleotides were purified as described in the user bulletin from ABI (no. 53; 1989) with minor modifications. In brief, synthesized oligonucleotides were incubated in 2 ml of ammonia solution at 55°C for 8 h to remove protecting groups. The crude deprotected oligonucleotides were purified on a OPC (oligonucleotide purification cartridge) column and then by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel with subsequent extraction from the gel with elution buffer (0.3 M ammonium acetate, 0.1 mM EDTA, 20 mM

Tris-HCl, pH 8.0) as described previously [68]. The sequences of oligonucleotides are indicated in Figures 2 and 4. Modified oligonucleotides were obtained from Oligo Service (Tsukuba, Japan). In addition, fluorescently labeled Dz3, which had 6-carboxyfluorescein at the 5' end, was synthesized as described previously [69,70]. Further purification of these oligonucleotides was based on polyacrylamide gel electrophoresis, as described above [68].

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

DNA templates for L6 *BCR-ABL* substrate mRNA and for the normal *ABL* substrate mRNA were synthesized chemically [28]. The DNA template for L6 *BCR-ABL* substrate mRNA 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 template for the normal *ABL* substrate mRNA extended from position 192 to position 283 of normal *ABL* cDNA [5–7]. Primers were also synthesized for each template, and each sense strand contained a T7 promoter. The sequences of the 5' and the 3' primers for L6 *BCR-ABL* substrate mRNA 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 mRNA were 5'-TAA TAC GAC TCA CTA TAG GGC TGT CCT CGT CCT CCA GCT G-3' and 5'-GCG GCT TCA CTC AGA CCC TGA GGC T-3'. Products of polymerase chain reactions (PCRs) were gel-purified. T7 transcription *in vitro* and gel-electrophoretic purification of the *ABL*, *BCR-ABL* mRNA substrates were performed as described elsewhere [28,68]. The lengths of the L6 *BCR-ABL* and *ABL* mRNA substrates were 121 and 92 nts, respectively.

Analysis of cleavage activities and specificities of DNA enzymes *in vitro*

Assays of activities and specificities of DNA enzymes were performed, in 25 mM MgCl₂ and 50 mM Tris-HCl (pH 8.0) at 37°C, under enzyme-saturating (single-turnover) conditions in which all the available substrate was expected to form a Michaelis-Menten complex, with high concentrations of the DNA enzyme and incubation for 10, 40 and 120 min or 1, 3 and 6 h as indicated in Figures 3 and 4. The substrate (*BCR-ABL* 121-mer substrate or normal *ABL* 92-mer) was labeled with [γ -³²P]-ATP by T4 polynucleotide kinase (Takara Shuzo, Kyoto, Japan). Each enzyme was incubated at 1 μ M with 2 nM 5'-³²P-labeled substrate. 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 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).

Kinetic analysis

Kinetic analysis of reactions catalyzed by DNA enzymes were performed with the *BCR-ABL* 121-mer substrate. Reaction rates were measured, in 25 mM MgCl₂ and 50 mM Tris-HCl (pH 8.0), under enzyme-saturating (single-turnover) conditions at 37°C, as described above. Cleavage rates were obtained from the slopes of the curves that represented the time-course of each reaction at the initial stage, and K_M and k_{cat} were calculated from Eadie-Hofstee plots. For determinations of K_M and k_{cat} , five different concentrations of enzyme (0.01, 0.02, 0.05, 0.25 and 1 μ M), spanning the K_M , were used and calculated values are summarized in Table 1. Cited values are averages of results from duplicate experiments (two sets of Eadie-Hofstee plots).

Luciferase assay

To measure luciferase activity, HeLa S3 cells were plated at 2×10^5 cells/well in a six-well plate 1 day prior to the (co)-transfection and incubated at 37°C in a CO₂ incubator. Plated HeLa S3 cells were washed twice with phosphate-buffered saline (PBS) and placed in

500 μ l of serum-reduced medium (OPTI-MEM I, Gibco BRL) before the (co)-transfection. In the assay shown in Figure 6, 3 μ g of each oligonucleotide (DNA enzyme, Antisense DNA etc.; final concentrations of each oligonucleotides were about 0.3 μ M) and 3 μ g of target expressing plasmid (pB2A2-luc or pABL-luc) were mixed with 6 μ l of Lipofectin reagent (Gibco-BRL, Rockville, MD) in 500 μ l of OPTI-MEM I medium, and incubated for 30 min at room temperature. After the incubation, the mixture was added gently to cells in OPTI-MEM I medium. After 2 h, the medium was replaced by the growth medium (DMEM) with 10% FCS and cells were cultured for another 10 h.

In the assay with high concentrations of oligonucleotides, the target expression plasmid and the oligonucleotide were added separately to cultured cells, in contrast to the experiments shown in Figure 6 (in which the oligonucleotides and the target-gene-expressing plasmid were simultaneously added to cells). The target-expressing plasmid (3 μ g) was mixed with 6 μ l Lipofectin in 500 μ l of OPTI-MEM I medium, and incubated for 30 min at room temperature. After the incubation, the mixture was added gently to cells in 500 μ l of OPTI-MEM I medium. After 2 h, HeLa S3 cells were washed twice with PBS and placed in 500 μ l of OPTI-MEM I for the second transfection. Ten nanomol of each oligonucleotide (final concentrations of each oligonucleotide were 10 μ M) was mixed with 6 μ l Lipofectin in 500 μ l of OPTI-MEM I medium, and the mixture was added to cells similarly. After incubation for 2 h, the medium was replaced by DMEM containing 10% FCS and cells were cultured for another 10 h.

Luciferase activity was measured with a PicaGene kit (Toyo-ink, Tokyo, Japan) as described elsewhere [29,56,71]. 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 was determined using a luminescent β -galactosidase genetic reporter system (Clontech, Palo Alto, CA) as described in the user bulletin [71].

RNAse H assay

The short RNA substrate S31, which is complementary to the binding sites of LDz3, was hybridized either with the DNA enzyme or with the antisense DNA prior to the RNAse H treatment. Hybridization reactions were carried out in a reaction buffer (20 mM Tris-HCl pH 7.5, 20 mM KCl, 10 mM MgCl₂, 1 mM DTT) containing 10 μ M of either the DNA enzyme or the antisense DNA, and 20 kcpm (< 2 nM) ³²P-labeled S31. The mixtures were heated at 90°C for 3 min and 10 units of RNAse inhibitor (TOYOBO, Osaka, Japan) was added. Samples were then incubated at 37°C for 6 h. Finally, the samples were incubated at 37°C with 0.001 unit of *Escherichia coli* RNAse H enzyme (Promega, WI) and quenched at specific time points. The substrate and the products of the reaction were separated by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel and were detected by autoradiography, as described above. The sequence of S31 was 5'-AUC AAU AAG GAA GAA GCC CUU CAG CGG CCA G-3'. The sequence of LDz3 was 5'-CTG GCC GCT GAA GGG GGC TAA CTA CAA CGA TTC TTC CTT ATT GAT-3'. The sequences of Anti-8 and Anti-15 were 5'-CTG GGC GCT GAA GGG-3' and 5'-CTG AAG GG-3', respectively.

Cell viability and apoptosis

BV173 and H9 cells were cultured at 5×10^7 cells/dish in RPMI 1640 medium containing 10% FCS at 37°C. Each oligonucleotide (DNA enzyme, 30 μ g) was mixed with 30 μ l Lipofectin in 800 μ l of OPTI-MEM I medium, and incubated for 30 min at room temperature. After the incubation, the mixture was added gently to cells which had been placed in OPTI-MEM I medium. After 2 h, the medium was replaced by growth medium (RPMI 1640) containing 10% FCS and cell viability was determined by the trypan blue exclusion test. Apoptosis was monitored as described previously [72], and cells were stained with 10 μ g/ml Hoechst 33342 (Nippon Gene Co., Toyama, Japan) for 15 min for studies of nuclear morphology. After washing and mounting in 90% glycerol/20 mM

Tris (pH 8.0)/0.1% *N*-propyl gallate, slides were examined under a fluorescent microscope (Nikon, Tokyo).

Northern blotting analysis

To detect cleaved *BCR-ABL* mRNA in BV173 cells, total RNA from BV173 cells which were transfected with each DNA enzyme as described above was isolated with ISOGENTM (Nippon Gene Co., Toyama, Japan). Total RNA (50 μ g per lane) was loaded on an agarose gel (FMC Inc., Rockland, ME) and, after electrophoresis, bands of RNA were transferred to a Hybond-NTM nylon membrane (Amersham Co., Buckinghamshire, UK). The membrane was probed with a synthetic oligonucleotide (5'-TCA GAT GCT ACT GGC CGC TGA AGG GCT T-3', complementary to the sequence of ABL mRNA) that had been labeled with ³²P by T4 polynucleotide kinase (Takara Shuzo Co., Kyoto). Prehybridization and hybridization were performed as described previously [29,56]. To detect cleavage by the DNA enzyme during the RNA isolation, total RNAs from BV173 cells (1×10^7 cells) that had not been transfected with the DNA enzyme were isolated with 1 ml of ISO-GENTM containing 20 μ g of MeODz3. Then, the RNAs were detected by Northern blotting analysis as described previously [29,56].

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