Targeting Sites within HIV-1 cDNA with a DNA-Cleaving Ribozyme†

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Received April 8, 1996; Revised Manuscript Received June 26, 1996⊗

ABSTRACT: A variant of the *Tetrahymena* ribozyme that efficiently cleaves single-stranded DNA under simulated physiological conditions [Tsang, J., & Joyce, G. F. (1994) *Biochemistry 33*, 5966−5973] was evaluated as a potential therapeutic agent on the basis of its ability to cleave synthetic oligonucleotide substrates corresponding to conserved target sites within HIV-1 cDNA. In order to increase the sequence selectivity of the ribozyme, its substrate recognition domain was extended from 6 to 12 nucleotides, allowing base pairing with substrate nucleotides that lie both upstream and downstream of the cleavage site. The sequence of the extended recognition domain could be changed to allow cleavage of a variety of different DNA targets. The ribozyme exhibited a high degree of sequence specificity, discriminating by a factor of 10^2 to more than 10^4 against substrates that form a single-base mismatch with the ribozyme's recognition domain. Mismatches that occurred close to the cleavage site led to a greater decrease in activity compared to those that occurred farther away.

Since the discovery of catalytic RNA molecules (Kruger et al., 1982; Guerrier-Takada et al., 1983), the notion of using ribozymes as therapeutic agents has attracted considerable interest [for recent review, see Christoffersen and Marr (1995)]. The ability of a ribozyme to recognize and cleave a specific RNA target (Zaug et al., 1986; Uhlenbeck, 1987; Altman, 1989; Hampel & Tritz, 1990) can be exploited to combat disease at the level of genetic information. In comparison to the related antisense oligonucleotide approach (Zamecnik et al., 1978; Goodchild et al., 1988), a ribozyme has the advantage of not only binding to the target in a sequence-specific manner but also cleaving it without the assistance of a cellular protein (Walder & Walder, 1988) or a tethered nucleolytic moiety (Dreyer & Dervan, 1985; Chu & Orgel, 1985). Thus, a ribozyme can be thought of as a sophisticated antisense agent. It includes both a recognition domain, which allows binding to the target, for example, via Watson−Crick base pairing, and a catalytic apparatus, which cleaves the bound target by either a hydrolytic or a transesterification mechanism.

High activity, specificity, and selectivity are important attributes of a therapeutic agent, each helping to diminish potential toxic side effects that might occur as a result of spurious interactions with other cellular macromolecules. In order to achieve high specificity, the ribozyme must distinguish between a target sequence and closely related sequences of the same length. In order to achieve high selectivity, the ribozyme must recognize a target sequence of sufficient length that it is unlikely to occur by chance among nontargeted cellular RNAs. Ideally, a ribozyme that is considered to be a general-purpose therapeutic tool should possess enough flexibility to allow its recognition domain to be adjusted to accommodate any desired RNA target without loss of catalytic activity, specificity, or selectivity.

Ribozymes that act as sequence-specific endoribonucleases can potentially be applied to the treatment of a broad range of disease states, the most actively pursued at present being cancer and viral infection. Several studies have demonstrated inhibition of oncogene function by a ribozyme (Koizumi et al., 1992; Snyder et al., 1993; Cantor et al., 1993). Efforts concerning antiviral applications have been directed primarily toward the inhibition of HIV-1 infection. In one of the first *in vivo* studies, an engineered hammerhead ribozyme directed against HIV-1 *gag* RNA was shown to reduce substantially the level of *gag*-associated p24 antigen (Sarver et al., 1990). Subsequent studies involving ribozymes directed against HIV-1 integrase RNA (Sioud & Drlica, 1991) or the U5 long terminal repeat (LTR) of HIV-1 (Weerasinghe et al., 1991; Ojwang et al., 1992) also gave promising results, encouraging further exploration of anti-HIV-1 ribozymes in a clinical setting. Research groups at the University of California, San Diego, and at the City of Hope in Duarte, CA, are expected to enter clinical trials with anti-HIV-I ribozymes in 1996 (Rawls, 1996).

All of the published studies to date concerning the use of ribozymes directed against disease-related targets have focused on RNA-catalyzed RNA cleavage reactions. Yet, engineered forms of group I and group II ribozymes also have the ability to cleave single-stranded DNA substrates (Tsang & Joyce, 1994; Griffin et al., 1995). This activity may be useful in targeting HIV-1, which passes through a single-stranded DNA intermediate as part of its replication cycle. The genome is packaged in the virion in the form of RNA, which is then reverse transcribed to yield a complementary DNA (cDNA). Recent studies have demonstrated that infectious virions typically contain partial or even full-length cDNA, generated by reverse transcription beginning at the U5 end of the viral genome (Trono, 1992; Lori et al., 1992). In addition, one study suggests that the existence of viral cDNA enhances the efficiency of intra- and interhost virus transmission (Zhang et al., 1993). These observations, coupled with the fact that cleavage of the cDNA may be

† Supported by research Grant AI 30882 from the National Institutes of Health.

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

S0006-2960(96)00845-8 CCC: $12.00

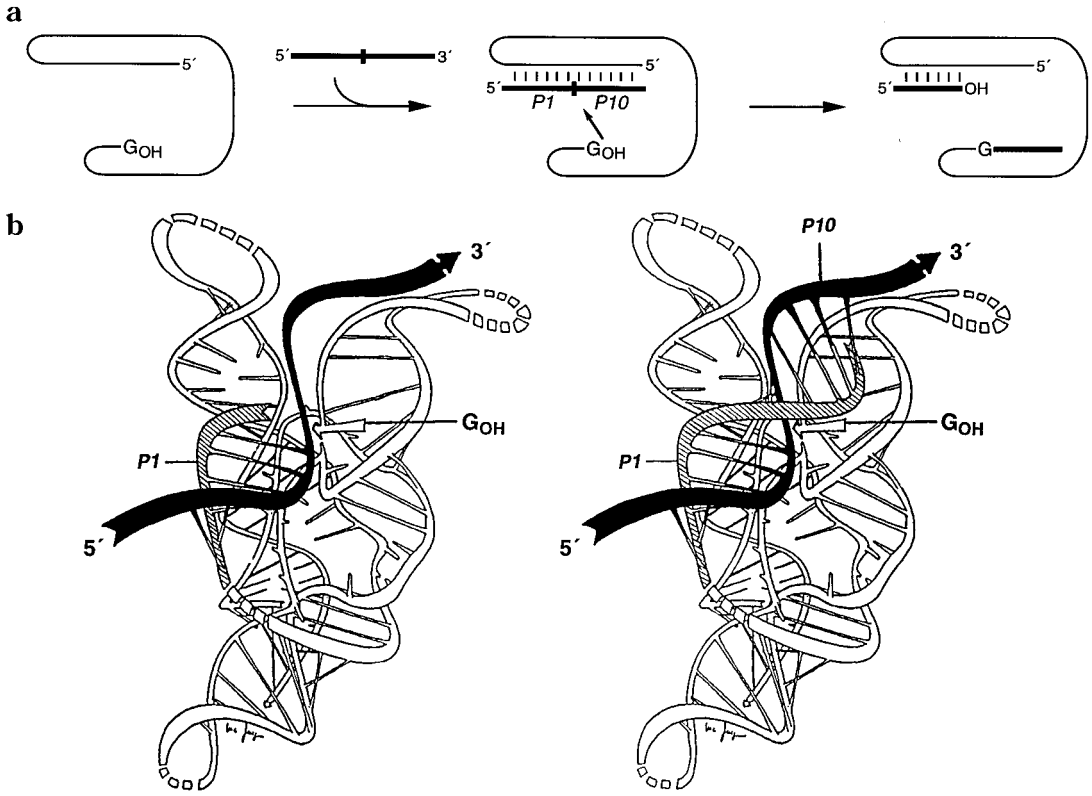

FIGURE 1: RNA-catalyzed DNA cleavage using the extended IGS. (a) Addition of the P10 domain allows substrate recognition through base-pairing interactions both upstream (P1 helix) and downstream (P10 helix) of the cleavage site. (b) Structural model of the catalytic core of a group I ribozyme (Michel & Westhof, 1990), showing either the P1 helix (left) or the P1+P10 helix (right) docked into position.

difficult to repair because there is no template to hold the cut ends in close proximity, suggest that HIV-1 cDNA may be an attractive target for ribozyme attack.

The *Tetrahymena* group I ribozyme, in addition to its efficient RNA cleavage activity, has a low level of DNA cleavage activity (Herschlag & Cech, 1990; Robertson & Joyce, 1990). Through the use of *in vitro* evolution techniques, Beaudry and Joyce (1992) and Tsang and Joyce (1994) obtained variants of this ribozyme that are able to cleave single-stranded DNA efficiently under simulated physiological conditions. One of the evolved ribozymes showed a 10^5-fold improvement in DNA cleavage activity compared to the wild type, with a second-order rate constant, k_{cat}/K_m, of 2.3×10^6 M^{-1} min^{-1} (Tsang & Joyce, 1994). Like the wild type, the evolved ribozyme recognizes its substrate through Watson−Crick base pairing between the internal guide sequence (IGS) of the ribozyme and substrate nucleotides that lie immediately to the 5′ side of the cleavage site (Zaug et al., 1986). In particular, the nucleotides 3′-GGGAGG-5′ of the IGS form a duplex (P1 helix) with the nucleotides 5′-CCCTCT-3′ of the substrate, utilizing the terminal G·T wobble pair to define the cleavage site. Cleavage proceeds via a transesterification mechanism, involving nucleophilic attack by the 3′-hydroxyl group of a guanosine residue that lies at the 3′ end of the ribozyme.

The goal of the present study was to investigate the possibility of using the evolved DNA-cleaving ribozyme more broadly to target various single-stranded DNA substrates, including those that are relevant to HIV-1 cDNA. In principle, one could target any DNA that allows Watson−Crick pairing with the IGS of the ribozyme (including the terminal G·T wobble pair) simply by changing the IGS in an appropriate manner. Successful alteration of substrate specificity has been reported for the RNA cleavage reaction catalyzed by the wild-type *Tetrahymena* ribozyme (Murphy & Cech, 1989; Campbell & Cech, 1995). Here we report that alteration of the IGS can lead to a substantial decrease in activity in the DNA cleavage reaction, as long as a recognition domain of only 6 nucleotides is utilized. However, extending the IGS so that it forms a total of 12 base pairs with the substrate, 6 upstream (P1 helix) and 6 downstream (P10 helix) from the cleavage site (Figure 1), restores DNA cleavage activity to a high level. High specificity is maintained, such that a single-base mismatch between ribozyme and substrate, either upstream or downstream from the cleavage site, results in markedly decreased cleavage activity.

With respect to substrate selectivity, a recognition domain of only 6 nucleotides is insufficient to ensure uniqueness of the substrate sequence among a large, heterogeneous pool of nucleic acids. Estimates of the shortest sequence that is likely to be unique in eukaryotic cells range from 11 to 15 nucleotides (Hélène et al., 1990; Herschlag, 1991; Woolf et al., 1992). Extension of the IGS from 6 to 12 nucleotides serves to enhance the selectivity of the ribozyme by increasing the number of nucleotides that must be recognized in order to obtain a high level of DNA cleavage activity.

In this study, we focused on four target sites within HIV-1 cDNA, all located within a highly conserved region of the genome (Figure 2). Each site consists of 12 nucleotides that form base pairs with the IGS of the ribozyme, including the G·T wobble that defines the cleavage site. Synthetic oligodeoxynucleotides were utilized as model substrates for these sites and in each case were shown to be cleaved by the appropriately configured ribozyme. One site, corresponding to a stretch of nucleotides within the U5 LTR

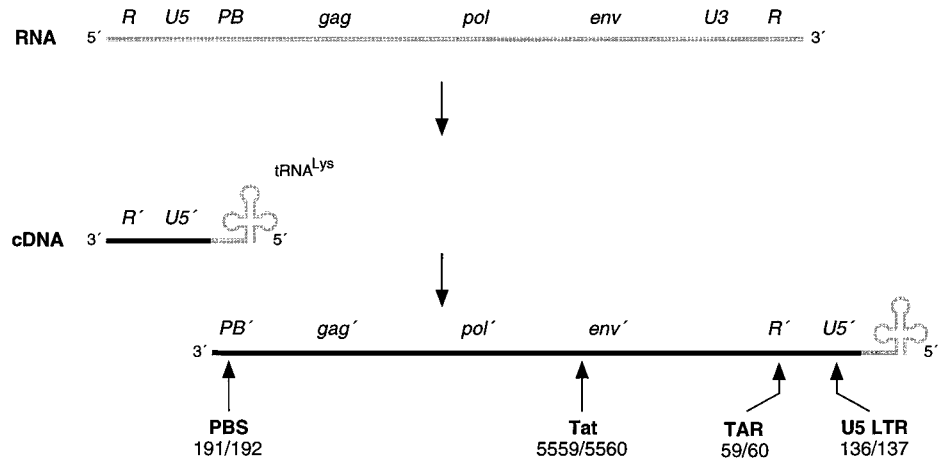

FIGURE 2: Location within HIV-1 cDNA of the four target sites that were examined in this study. Synthesis of cDNA proceeds in two steps. First, a tRNA primer is extended through the *U5* and *R* regions; then, the 3′ terminus of the partial-length cDNA "jumps" to the *R* region at the opposite end of the genomic RNA, and cDNA synthesis continues through the *U3*, *env*, *pol*, *gag*, and *PB* regions. The four target sites are shown in the lower portion of the figure, indicating the nucleotide position at which cleavage occurs. Nucleotide numbers refer to the corresponding genomic RNA, as reported by Ratner et al. (1985).

region of HIV-1 cDNA, was chosen for detailed analysis of the specificity of the DNA cleavage reaction.

MATERIALS AND METHODS

Materials. Synthetic RNA oligonucleotides were purchased from Oligos Etc., and DNA oligonucleotides were from Operon Technologies. Nucleoside triphosphates (NTPs) were obtained from Pharmacia and deoxynucleoside triphosphates (dNTPs) from U.S. Biochemical, and [γ-^{32}P]ATP was from ICN Radiochemicals. *Thermus aquaticus* DNA polymerase was cloned from total genomic DNA (Jaeger, Wright, and Joyce, unpublished results) and purified according to the protocol of Pluthero (1993). T7 RNA polymerase was prepared from the cloned gene (Davanloo, 1984) and purified by phosphocellulose column chromatography followed by anion-exchange chromatography using a Biocad FPLC apparatus (Raillard and Joyce, unpublished results). T4 polynucleotide kinase was obtained from New England Biolabs, inorganic pyrophosphatase from Sigma, and RNase-free DNase I from Boehringer Mannheim. Nensorb 20 reverse-phase chromatography columns were purchased from DuPont, and prepacked Sephadex G-25 columns were from Pharmacia.

Purification of Synthetic Oligonucleotides. Synthetic oligonucleotides (50−500 nmol) were purified by denaturing polyacrylamide gel electrophoresis, eluted from the gel with high-salt buffer (200 mM NaCl) either overnight at 23 °C or for 4 h at 37 °C, precipitated with ethanol, and desalted on a Sephadex G-25 column. Eluate fractions containing the oligonucleotide were identified by UV visualization, pooled, and precipitated with ethanol. The pellets were redissolved in 1 mM EDTA and 10 mM Tris (pH 8.0 for DNA, pH 7.5 for RNA) and stored at −20 °C.

Preparation of Labeled Synthetic Substrates. Synthetic DNA substrates were phosphorylated in a 20 μL reaction mixture containing 20 pmol of oligonucleotide, 22 pmol of [γ-^{32}P]ATP (7 μCi/pmol), 5 mM $MgCl_2$, 25 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES, pH 9.0), 3 mM dithiothreitol (DTT), and 1.25 units/μL T4 polynucleotide kinase. After incubation at 37 °C for 30 min, the reaction was quenched with EDTA, and the labeled oligonucleotides were purified by electrophoresis in a denaturing 20% polyacrylamide gel, visualized by autoradiography, eluted with high-salt buffer, and desalted by reverse-phase chromatography on a Nensorb column.

Changing Internal Guide Sequence by PCR. A mutagenic primer was designed that has the same sense as the ribozyme and contains the promoter sequence for T7 RNA polymerase (underlined), the novel IGS, and 18 nucleotides immediately downstream of the IGS: 5′-CTGCAGAATTCTAATAC-GACTCACTATA-(IGS sequence)-AAAAGTTATCAG-GCATGC-3′. The other primer was complementary to 20 nucleotides at the 3′ end of the ribozyme: 5′-CGAG-TACTCCAAAACTAATC-3′. When the altered IGS contained no or only one guanosine at the 5′ end, one or two additional guanosines that were not part of the substrate recognition sequence were added to ensure efficient *in vitro* transcription. The parent ribozyme (G27, #48; Tsang & Joyce, 1994) contains a G → A mutation at position 44 that was reverted to G by the mutagenic primer. Control experiments demonstrated that this mutation did not alter the catalytic behavior of the ribozyme under any of the conditions that were studied. PCR amplification was carried out in a 100 μL volume containing 10−20 fmol of ribozyme-encoding DNA, 20 pmol of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 0.1% gelatin, each dNTP (0.2 mM), and 0.05 unit/μL Taq DNA polymerase, employing 30 cycles of 92 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min.

Preparation of Ribozymes. The PCR-amplified double-stranded DNA was purified by phenol extraction followed by ethanol precipitation and redissolved in 1 mM EDTA and 10 mM Tris (pH 8.0). *In vitro* transcription was carried out in a 50 μL reaction mixture containing 5 pmol of DNA, 15 mM $MgCl_2$, 2 mM spermidine, 50 mM Tris (pH 7.5), 5 mM DTT, each NTP (2 mM), 0.005 unit/μL inorganic pyrophosphatase, and 25 units/μL T7 RNA polymerase, which was incubated at 37 °C for 2 h. The double-stranded DNA was digested by incubation at 37 °C for 30 min in the presence of 0.5 u/μL RNase-free DNase I, and the reaction was quenched with EDTA. The RNA was purified by phenol extraction and ethanol precipitation, followed by electrophoresis in a denaturing 5% polyacrylamide gel. Ribozymes were visualized by UV shadowing, eluted from the gel, and

further purified on a reverse-phase Nensorb column. Their concentration was determined spectrophotometrically, as described previously (Zaug et al., 1988). Ribozymes were stored at −20 °C in buffer containing 0.1 mM EDTA and 1 mM Tris (pH 7.5).

Kinetic Analysis. RNA-catalyzed DNA cleavage reactions were performed as described previously (Tsang & Joyce, 1994). Synthetic oligodeoxynucleotides were used as model substrates. Those nucleotides that were recognized by the IGS of the ribozyme are indicated in bold throughout the text. Unlabeled ribozyme and 5′-^{32}P-labeled substrate were preincubated separately at 37 °C for 15 min in a buffer containing 10 mM $MgCl_2$, 30 mM 4-(2-hydroxyethyl)-piperazine-1-propanesulfonic acid (EPPS, pH 7.5), and 40 μg/mL bovine serum albumin (BSA), and the reaction was initiated by mixing. Aliquots (2.5−4 μL) were withdrawn at specific times and the reactions quenched by addition of an equal volume of an ice-cold mixture containing 8 M urea, 100 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue, 10% SDS, 9 mM Tris-borate (pH 8.3), and 20% sucrose. Cleavage product was separated from unreacted substrate by denaturing polyacrylamide gel electrophoresis, and the fraction cleaved was quantitated with a PhosphorImager model SI (Molecular Dynamics).

The parameters k_{cat} and K_m for all but two of the ribozymes were determined under conditions with ribozyme in large excess over substrate, using labeled substrate at a concentration of 1−5 nM. Values for k_{obs} were obtained for the DNA-cleavage reaction in the presence of various concentrations of ribozyme, based on 5 data points generated over the first 5−15% of the reaction. A plot of k_{obs} versus ribozyme concentration produced a hyperbolic curve that was fit to the equation: $k_{obs} = k_{cat}[E]/([E] + K_m)$, which holds true when $[S] << K_m$, assuming that binding of the ribozyme and substrate has reached pre-equilibrium. The kinetic parameters k_{cat} and K_m and their standard errors were calculated by non-linear regression analysis using the Marquardt-Levenberg algorithm (SigmaPlot).

The kinetic properties of the U5 LTR_{P1} ribozyme (IGS = 5′-GGAGAU-3′) and the U5 $LTR_{P1}(U_{27} \rightarrow G)$ ribozyme (IGS = 5′-GGAGAG-3′) were determined under conditions with substrate in large excess over ribozyme. The K_m value for these ribozymes was greater than 10 μM, and at ribozyme concentrations approaching saturation, effects of aggregation and precipitation led to irreproducible results. Thus, the ribozyme concentration was held constant at 1.5 μM and the concentration of substrate was varied, employing a trace amount of labeled substrate that was mixed with a large amount of unlabeled material. Initial rates (v_o) were determined over the first 10 min of the reaction in the presence of varying concentrations of substrate, and the data were fit to the Michaelis−Menten equation: $v = k_{cat}[E_o]/(K_m + [S])$. The individual kinetic parameters were calculated by nonlinear regression analysis as described above.

Determination of Binding Constants for RNA−DNA Heteroduplexes. The equilibrium dissociation constant, K_D, for the RNA−DNA duplexes 5′-GGUAACUGGAGAU-3′•5′-ATCTC-CAGTTACC-3′ and 5′-GGAGAU-3′•5′-ATCTCC-3′ were determined as described previously (Tsang & Joyce, 1994), on the basis of gel-shift analysis in a native polyacrylamide gel employing 5′-^{32}P-labeled DNA and varying concentrations of unlabeled RNA. The RNA and DNA molecules were preincubated separately at 37 °C for 10 min in a buffer containing 10 mM $MgCl_2$, 30 mM EPPS (pH 7.5), and 40 μg/mL BSA and then mixed and equilibrated at 37 °C for 1 h. The gel and running buffer also contained 10 mM $MgCl_2$ and 30 mM EPPS (pH 7.5) and were preheated to 37 °C prior to loading. Immediately before the gel was loaded, an aliquot of loading buffer (20% v:v), containing 0.3% bromophenol blue, 9% glycerol, 10 mM $MgCl_2$, 30 mM EPPS (pH 7.5), and 40 μg/mL BSA, was added to the mixture. No more than 10 s elapsed between addition of the loading buffer and the time the sample entered the gel. All manipulations were performed in an environmental room at 37 °C. The fraction of bound (gel-shifted) DNA molecules was quantitated with a PhosphorImager, with measurements obtained over a broad range of RNA concentrations. The data were fit to the equation $f_{bound} = [RNA]/([RNA] + K_D)$, as described above.

RESULTS

Generality of a DNA-Cleaving Ribozyme. Tsang and Joyce (1994) carried out 27 "generations" of *in vitro* evolution to obtain variants of the *Tetrahymena* ribozyme that efficiently cleave single-stranded DNA under simulated physiological conditions (10 mM $MgCl_2$, pH 7.5, and 37 °C). A typical ribozyme isolated from the evolved population contained 17 mutations relative to the wild type and cleaved the target substrate 5′-GG**CCCTCT**AAATAAATA-3′ with a catalytic efficiency, k_{cat}/K_m, of 1.1×10^6 M^{-1} min^{-1}. We began with this evolved ribozyme and examined its ability to cleave two different substrates that contain the same target sequence, 5′-**CCCTCT**-3′, joined to two different 3′-terminal sequences, 5′-AAATAAATA-3′ and 5′-AGTTACA-3′, located downstream from the scissile bond. The two substrates were cleaved with comparable efficiency and nearly identical values for both k_{cat} (0.48 and 0.49 min^{-1}, respectively) and K_m (470 and 430 nM, respectively). This confirms that the downstream portion of the substrate, which is not recognized by the IGS, does not significantly affect the catalytic activity of the ribozyme.

We then changed the guide sequence of the ribozyme, using an appropriately designed mutagenic primer during PCR amplification, followed by *in vitro* transcription (see Materials and Methods). The various combinations of IGS and substrate that were tested and the corresponding second-order rate constant of the ribozyme-catalyzed reaction are shown in Figure 3. A change of only one nucleotide in the IGS, together with a compensatory change in the substrate, resulted in a substantial decrease in catalytic activity. For example, changing the rG•dC pair that lies 6 nucleotides upstream from the cleavage site to an especially weak rU•dA pair resulted in more than a 50-fold decrease in catalytic activity. Changing the rG•dC pair that lies 5 nucleotides upstream from the cleavage site to a rA•dT pair reduced activity 370-fold. The combination of these two alterations was even more detrimental, with catalytic efficiency reduced 830-fold compared to that of the original IGS•substrate pairing. The doubly altered target sequence, 5′-**ATCTCT**-3′, corresponds to a 6-nucleotide segment within the U5 LTR region of HIV-1 cDNA (U5 LTR_{P1}) and was cleaved with a k_{cat}/K_m of 1.2×10^3 M^{-1} min^{-1} in the RNA-catalyzed reaction.

Comparison of U5 LTR_{P1} and U5 LTR_{P1+P10} Ribozymes. The loss of activity that resulted from alteration of the

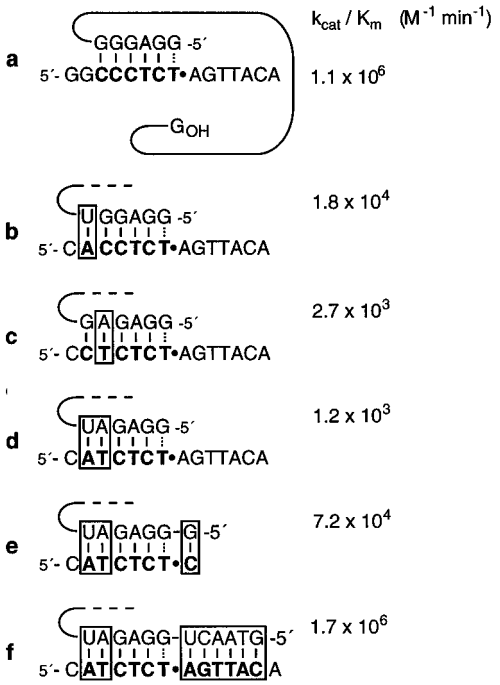

FIGURE 3: Various IGS·substrate complexes and corresponding values for k_{cat}/K_m in the RNA-catalyzed DNA cleavage reaction. Substrate nucleotides that are recognized by the IGS are shown in bold, and base-pair changes are indicated by boxes. (a) Original IGS·substrate combination. (b) Replacement of rG·dC by rU·dA at a position six nucleotides upstream from the cleavage site. (c) Replacement of rG·dC by rA·dT at a position five nucleotides upstream from the cleavage site. (d) Combination of the two changes in b and c (U5 LTR_{P1} ribozyme). (e) Extension of the IGS by a single G·C pair (U5 LTR_{P1+C} ribozyme). (f) Extension of the IGS by six base pairs (U5 LTR_{P1+P10} ribozyme).

original IGS to the U5 LTR_{P1} sequence could be offset by extending the IGS so that it paired with one or more nucleotides of the substrate that lie downstream from the cleavage site. Addition of a single base pair to the IGS·substrate interaction (U5 LTR_{P1+C}) increased catalytic activity 60-fold. Addition of 6 base pairs (U5 LTR_{P1+P10}), resulting in a recognition domain of 6 base pairs upstream and 6 downstream from the cleavage site, fully restored DNA cleavage activity, with a value for k_{cat}/K_m of 1.7×10^6 M^{-1} min^{-1}.

The individual kinetic parameters k_{cat} and K_m were determined in single-turnover reactions, either with ribozyme in large excess over substrate in the case of the U5 LTR_{P1+P10} ribozyme (Figure 4a) or with substrate in large excess over ribozyme in the case of the U5 LTR_{P1} ribozyme (Figure 4b). These data reveal that the substantial increase in catalytic activity of the P1+P10 ribozyme compared with that of the P1 ribozyme is due primarily to enhanced substrate binding, assuming that K_m reflects K_D (Table 1). Addition of the P10 domain led to only a 10-fold increase in k_{cat}, but more than a 100-fold decrease in K_m. Similar but intermediate effects were observed with the U5 LTR_{P1+C} ribozyme, which contains a P10 domain comprised of only a single base pair.

Alteration of IGS Allows Cleavage of Various DNA Substrates. Contiguous stretches of 12 nucleotides within the U5 LTR, primer binding site (PBS), transactivation response (TAR) element, and transactivation protein (Tat) region of HIV-1 cDNA were chosen as targets for ribozyme-catalyzed cleavage (Figure 2). The IGS of the ribozyme was designed to bind the substrate through Watson−Crick pairing

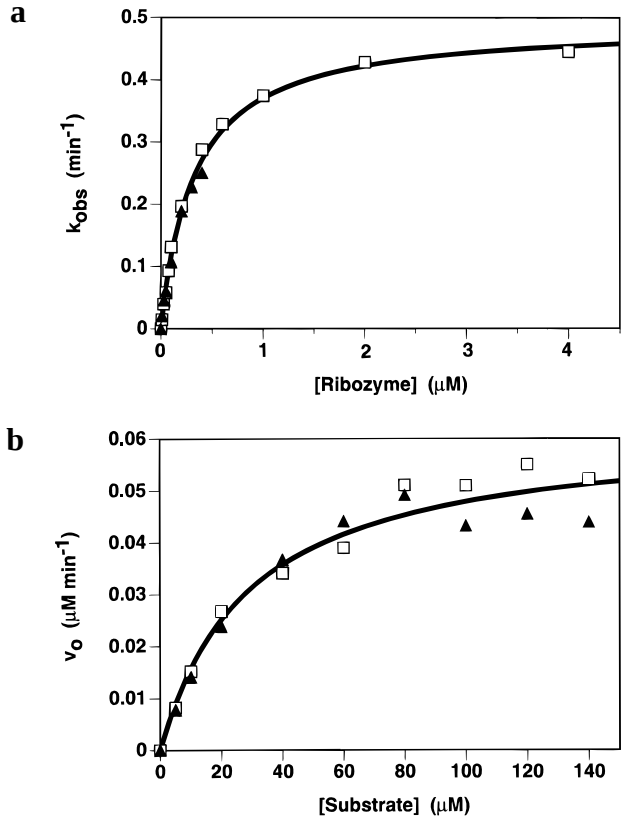

FIGURE 4: Kinetic data obtained under single-turnover conditions. (a) Analysis of the U5 LTR_{P1+P10} ribozyme was carried out using a large excess of ribozyme and trace amounts of 5′-^{32}P-labeled substrate. Data from two independent experiments are shown. The parameters k_{cat} and K_m were obtained using the formula $k_{obs} = k_{cat}[E]/([E] + K_m)$. (b) Analysis of the U5 LTR_{P1} ribozyme was carried out using a large excess of substrate and a fixed concentration of ribozyme. Data from two independent experiments are shown and were fit to the equation $v = k_{cat}[E_o]/(K_m + [S])$.

involving 6 nucleotides upstream and 6 downstream from the cleavage site. The kinetic parameters for cleavage of the various substrates are summarized in Table 2.

The ribozyme that recognizes and cleaves a 12-nucleotide stretch within the U5 LTR region of HIV-1 cDNA (U5 LTR_{P1+P10}) showed the greatest activity, with a second-order rate constant, k_{cat}/K_m, of 1.7×10^6 M^{-1} min^{-1}. All of the other ribozymes were somewhat reduced in catalytic efficiency, the least active being the ribozyme directed against the Tat target, with a value for k_{cat}/K_m of 6.1×10^4 M^{-1} min^{-1}. Examination of individual kinetic parameters revealed that the value for K_m did not vary significantly among the four ribozymes, in all cases being in the range of 100−400 nM. The differences in catalytic efficiency were largely attributable to differences in k_{cat}, with values ranging from 0.013 min^{-1} for the ribozyme directed against the Tat target to 0.48 min^{-1} for the ribozyme directed against the U5 LTR target.

Effect of Mismatches between IGS and Substrate. We evaluated the sequence specificity of substrate recognition by the ribozyme by examining the effect of base mismatches between the IGS and substrate in the case of the U5 LTR target sequence. Values for k_{cat}/K_m were obtained under single-turnover conditions using trace amounts of 5′-^{32}P-labeled substrate and a large excess of ribozyme (Figure 5). The numbering of nucleotide positions reflects the distance from the scissile bond, positive for nucleotides in the P10 helix and negative for those in the P1 helix.

Table 1: Kinetic Properties of Various Ribozyme-Substrate Pairs

	IGS sequence	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\times 10^5$ M^{-1} min^{-1})	ΔG°_{37} calcd[a] (kcal mol^{-1})	K_D calcd[b] (μM)
original IGS	5′-GGAGGG-3′	0.45 ± 0.04	0.43 ± 0.07	11 ± 3	−7.1	9.9
U5 LTR_{P1}	5′-GGAGAU-3′	0.046 ± 0.002	36 ± 6	0.012 ± 0.003	−3.7	2500
U5 $LTR_{P1(U_{27}\rightarrow G)}$	5′-GGAGAG-3′	0.037 ± 0.005	14 ± 1	0.027 ± 0.001	−4.4	790
U5 $LTR_{P1(A_{26}\rightarrow G)}$	5′-GGAGGU-3′	0.048 ± 0.001	2.6 ± 0.2	0.18 ± 0.02	−5.8	82
U5 LTR_{P1+C}	5′-GGGAGAU-3′	0.34 ± 0.02	4.7 ± 0.4	0.7 ± 0.1	−5.2	220
U5 LTR_{P1+P10}	5′-GUAACUGGAGAU-3′	0.48 ± 0.01	0.29 ± 0.01	17 ± 1	−10.3	0.055

[a] Based on thermodynamic values obtained by Sugimoto et al. (1995) for DNA−RNA duplexes, with the ΔG° value for a rG·dT wobble pair substituted by the value reported for a rG·rU wobble (Freier et al., 1986). [b] Calculated using the formula $\Delta G = -RT \ln K_D$, with $R = 1.987$ cal K^{-1} mol^{-1} and $T = 310.15$ K.

Table 2: Kinetic Properties of Ribozymes Targeting Various HIV-1 cDNA Sequences

target	sequence[a]	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (10^6 M^{-1} min^{-1})
U5 LTR	5′-ATCTCTAGTTAC-3′	0.48 ± 0.01	0.29 ± 0.01	1.70 ± 0.08
PBS	5′-CCTGTTCGGGCG-3′	0.033 ± 0.003	0.11 ± 0.03	0.31 ± 0.13
TAR	5′-AGCAGTGGGTTC-3′	0.043 ± 0.004	0.36 ± 0.06	0.12 ± 0.33
Tat	5′-TAGGCTGACTTC-3′	0.013 ± 0.001	0.22 ± 0.04	0.06 ± 0.01

[a] All substrates contained a C residue at the 5′ end and an A residue at the 3′ end that were not part of the sequence that was recognized by the IGS.

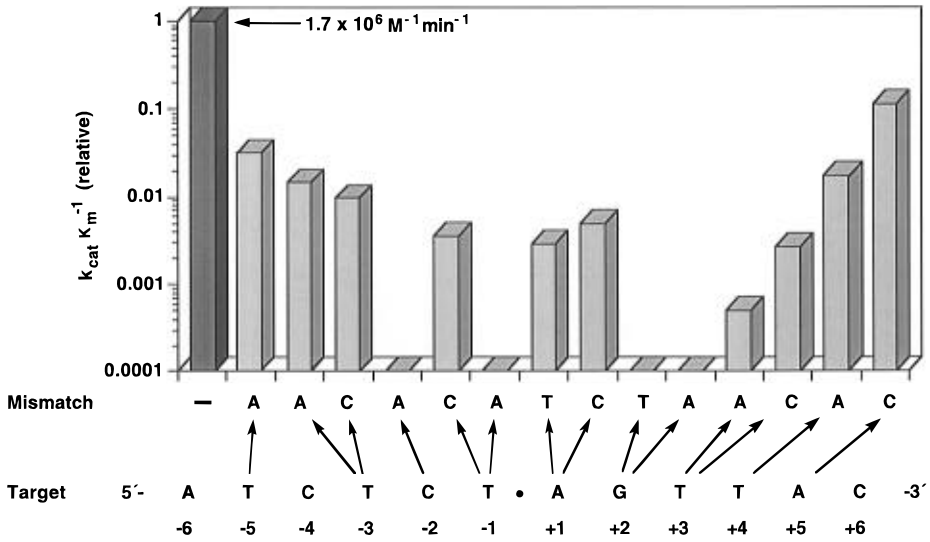

FIGURE 5: Bar graph showing relative catalytic efficiency of various IGS·substrate pairs in the context of the U5 LTR_{P1+P10} ribozyme, comparing the fully matched substrate (−) with substrates that form a single-base mismatch with the IGS. The value for k_{cat}/K_m for the fully matched substrate was assigned a relative value of 1. The numbering of nucleotide positions reflects the distance from the cleavage site, positive for nucleotides in the P10 helix and negative for those in the P1 helix. Values for k_{cat}/K_m significantly below 10^2 M^{-1} min^{-1} could not be determined.

The overall tendency is that the closer the mismatch lies to the scissile bond, the more detrimental it is to cleavage activity. This observation is particularly true for mismatches in the P10 helix, with the notable exception of position +1. Mismatches at the +2 position eliminated activity almost completely, with cleavage products still detectable, but corresponding to k_{cat}/K_m values substantially lower than 10^2 M^{-1} min^{-1}. In the P1 helix, the distance of the mismatch to the scissile bond appears to be less critical. Mismatches at the −3 and −5 positions reduced catalytic activity by approximately 70- and 30-fold, respectively, compared with that of the fully matched substrate. Positions −1 and −2 were far more sensitive to mismatch; even at high ribozyme concentrations (≥1 μM) and long incubation times (≥1 h), no cleavage product was detected. Position −1 is the site of the G·T wobble pair between the IGS and substrate that defines the cleavage site. Changing the G·T wobble to a G·A mismatch resulted in a complete loss of catalytic activity. However, substitution of the G·T wobble by a G·C Watson−Crick pair resulted in some residual activity, with k_{cat}/K_m decreased by about 300-fold.

We also examined the effect of double mismatches between the IGS and substrate by combining a mismatch in the P1 helix with one in the P10 helix. In one case, we combined an A·A mismatch at the −5 position, which alone reduced activity by ∼30-fold, and a U·C mismatch at the +5 position, which alone reduced activity by ∼10-fold. Together, these two mutations resulted in a 150-fold decrease in activity. A similar multiplicative effect was observed when an A·A mismatch at position −5 and an A·A mismatch at position +4 were combined to produce a double mismatch that exhibited a 5000-fold reduction in activity. In summary, our data show that the ribozyme is able to discriminate against alternative DNA substrates that differ from the target sequence by only one or two nucleotides, especially when the mismatch lies close to the cleavage site.

We determined individual k_{cat} and K_m values for the cleavage reaction with three different singly mismatched

Scheme 1

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\bullet}S \overset{k_{dock}}{\rightleftharpoons} E{\bullet}S' \overset{k_{chem}}{\rightleftharpoons} E{\bullet}P$$

substrates. An A·A mismatch at position −3 reduced k_{cat} by 13-fold (0.037 min) and increased K_m by 5-fold (1.5 μM) compared to those values for the reaction with the fully matched substrate. An A·A mismatch at position −5 resulted in a k_{cat} of 0.11 min^{-1} and a K_m of 2.0 μM, while an A·A mismatch at position +4 resulted in a k_{cat} of 0.044 min^{-1} and a K_m of 1.4 μM. Thus, in all three cases, effects on both k_{cat} and K_m contribute to the ribozyme's ability to discriminate against mismatched substrates.

DISCUSSION

We have explored the possibility of using a DNA-cleaving ribozyme, an evolved variant of the *Tetrahymena* ribozyme, as a therapeutic tool by directing it against target sequences that occur within conserved regions of HIV-1 cDNA. In principle, one could apply this ribozyme in a general-purpose manner by modifying its recognition domain so as to recognize various single-stranded DNA targets. However, the generality of the recognition domain must not be obtained at the expense of catalytic activity, sequence specificity, or sequence selectivity.

RNA cleavage by the wild-type *Tetrahymena* ribozyme was shown to proceed with comparable efficiency when the IGS was changed to recognize various target substrates (Murphy & Cech, 1989; Campbell & Cech, 1995). Coincidentally, Campbell and Cech examined the cleavage of an RNA version of the same sequence that we targeted with the DNA-cleaving U5 LTR_{P1} ribozyme. They obtained a value for k_{cat}/K_m in the RNA cleavage reaction of 5×10^7 M^{-1} min^{-1} for both the original substrate, 5′-**CCCUCU**·AA-3′, and the altered substrate, 5′-**AUCUCU**·AC-3′. In contrast, the evolved DNA-cleaving ribozyme was highly sensitive to alteration of the IGS, cleaving the original substrate, 5′-GG**CCCTCT**·AGTTACA-3′, 830-fold more efficiently than the altered substrate, 5′-C**ATCTCT**·AGTTACA-3′. This marked reduction of catalytic activity must be attributed to the fact that two rG·dC pairs that were involved in the original IGS−substrate interaction were replaced by a rU·dA and a rA·dT pair. Either replacement alone resulted in substantially decreased activity, and the two together were far more deleterious (Figure 3).

There are two possible explanations for the greater sensitivity to substrate sequence of the evolved DNA-cleaving ribozyme compared to the wild-type RNA-cleaving ribozyme. The DNA-cleaving ribozyme was obtained after 27 generations of *in vitro* evolution utilizing the original IGS, 5′-GGAGGG-3′. Over the course of evolution, the ribozyme may have developed a preference for that particular sequence, in some way becoming "addicted" to it. Alternatively, the ribozyme may have become dependent on the highly stable base-pairing interaction that occurs when a G-rich IGS binds a C-rich substrate, irrespective of the particular nucleotides that are involved. In order to distinguish between these two possibilities, it is important to consider the mechanism by which the ribozyme recognizes its substrate.

Binding of an RNA substrate by the wild-type ribozyme occurs in two steps (Scheme 1). First, the IGS of the ribozyme forms Watson−Crick pairs with complementary nucleotides of the substrate. Then the IGS·substrate duplex (P1 helix) is docked into the catalytic core of the ribozyme through tertiary interactions. Studies conducted by Bevilacqua et al. (1992) and Herschlag et al. (1992) indicate that the first step involves base pairing alone. The second-order rate constant, k_{on}, for formation of the P1 helix is thought to be limited by the rate of helical nucleation, which is largely independent of nucleotide sequence and length beyond the trimer. Thus, differences in the stability of the P1 helix are reflected in differences in the rate of duplex dissociation, k_{off}.

The second step of binding involves docking of the P1 helix into the catalytic core. Docking provides additional binding energy for the ribozyme-substrate complex through tertiary interactions (Sugimoto et al., 1989; Pyle et al., 1990). Several contacts between the P1 helix and specific residues in the catalytic core have been identified, for example, involving the exocyclic amine of the guanine that forms the G·U wobble pair (Strobel & Cech, 1995; Strobel & Cech, 1996). Additional binding energy is provided by hydrogen-bonding interactions involving 2′-hydroxyl groups of the sugar−phosphate backbone of both the IGS (Strobel & Cech, 1993) and substrate strands (Pyle et al., 1991, 1992; Bevilacqua & Turner, 1991). Of course, all interactions that involve 2′-hydroxyls of the substrate must be absent when the ribozyme binds a DNA substrate, resulting in significantly decreased docking energy and consequently poor catalytic activity (Herschlag & Cech, 1990).

According to the results obtained by Campbell and Cech (1995), cleavage of an RNA substrate by the wild-type ribozyme is not affected when the dissociation rate for the first step of binding is made faster by employing a less stable P1 helix. This suggests that docking of the IGS·RNA substrate duplex is sufficiently fast to outcompete the dissociation rate of the duplex. This conclusion is in accordance with the observation that, under single-turnover conditions, the docking and cleavage steps are sufficiently fast to make helical nucleation the rate-limiting step of the reaction (Herschlag, 1993). Available data concerning the docking rate (Bevilacqua et al., 1992, 1994; Herschlag, 1992; Li et al., 1995) are consistent with the notion that the rate of docking greatly exceeds the rate of duplex dissociation in the RNA cleavage reaction catalyzed by the wild-type ribozyme.

In the DNA cleavage reaction with the wild-type ribozyme, the docking process is impaired and is potentially rate-limiting for substrate binding and cleavage. As a result of *in vitro* evolution, the ribozyme has been forced to compensate for the loss of 2′-hydroxyl contacts with the DNA substrate, presumably by developing novel docking interactions. The wild-type ribozyme binds the product 5′-GG**CCCTCT**-3′ with a K_D of 30 μM, whereas the evolved ribozyme binds the same product with a K_D of 4 nM (10 mM $MgCl_2$, pH 7.5, and 37 °C; Tsang & Joyce, 1994). The novel docking interactions, however, were developed in the context of a P1 helix that has a slow dissociation rate so that docking need not be very fast in order to exceed the rate of duplex dissociation. In this way, the evolved ribozyme may have become adapted to a highly stable Watson−Crick pairing between the IGS and substrate.

To examine this hypothesis, we calculated the dissociation constant, K_D, for various RNA−DNA heteroduplexes that correspond to Watson−Crick pairing between the IGS and

a DNA substrate. Relying on thermodynamic measurements obtained by Sugimoto et al. (1995), the free energy (ΔG°_{37}) for RNA−DNA duplex formation was calculated, and the dissociation constant was calculated using the formula: $-RT \ln K_D = \Delta G$ (Table 1). Because the predicted ΔG°_{37} value is only an approximation of the true value, we verified two of the calculated dissociation constants by performing a gel-shift analysis under our standard reaction conditions (10 mM $MgCl_2$, pH 7.5, and 37 °C; see Materials and Methods). The experimentally determined values for K_D were in good agreement with the predicted ones: 5′-GUAACUGGAGAU-3′•3′-CATTGACCTCTA-5′ and 5′-GGAGAU-3′•3′-CATTGACCTCTA-5′ exhibited K_D values of 32 ± 6 nM and 2.5 ± 0.4 mM, respectively while the calculated values were 55 nM and 2.5 mM, respectively. Comparing the calculated values for K_D with the experimentally determined values for K_m (Table 1), it is apparent that an increase in K_D, reflecting a faster dissociation rate of the P1 helix, correlates with an increase in K_m, and thus with reduced catalytic efficiency. The increase in K_m that results from alteration of the IGS can be compensated for by extending the IGS to form additional base pairs with substrate nucleotides that lie downstream from the cleavage site, as in the case of the U5 LTR_{P1+C} and U5 LTR_{P1+P10} ribozymes.

Further support for the hypothesis that the evolved DNA-cleaving ribozyme has become adapted to the high stability of the IGS•substrate duplex rather than to particular nucleotides comes from kinetic data obtained for the various P1+P10 ribozymes (Table 2). Despite marked differences in the sequence of their IGS, all of these ribozymes have a K_m that lies within the narrow range of 100−400 nM. Thus, differences in their catalytic efficiency are largely attributable to differences in k_{cat}. There is no obvious correlation between the sequence of the IGS and k_{cat}. Sequences that are more distantly related to the original IGS are not necessarily associated with a lower k_{cat}. For example, two ribozymes that had different P1 domains (IGS_{P1} = 5′-GGAGAU-3′ or 5′-GCCGCU-3′) and the same P10 domain (IGS_{P10} = G) had nearly identical k_{cat} values (0.34 and 0.37 min^{-1}, respectively), even though the former is more closely related to the original ribozyme (IGS_{P1} = 5′-GGAGGG-3′). In the case of the U5 LTR_{P1} ribozyme, we found that introduction of a P10 helix (U5 LTR_{P1+C} and U5 LTR_{P1+P10} ribozymes) restored k_{cat} to a level comparable to that of the original ribozyme, even though the sequence of the P1 helix was very different from that of the original ribozyme.

Addition of the P10 recognition domain to the DNA-cleaving ribozyme not only serves to maintain high catalytic efficiency by providing sufficient duplex stability for the first step of substrate binding but also enhances sequence selectivity. With the cleavage site positioned in the middle of an extended duplex, the ribozyme must recognize substrate nucleotides that lie both upstream and downstream of the scissile bond. High sequence specificity is maintained in both the P1 and P10 domains, with a single-base mismatch between IGS and substrate resulting in a 10^2- to more than 10^4-fold reduction in catalytic activity, depending on its distance from the cleavage site and the particular nucleotides that are involved (Figure 5). In general, mismatches close to the cleavage site resulted in a marked reduction in activity, while those farther away resulted in a more modest reduction. This behavior is similar to what has been described for the RNA cleavage reaction catalyzed by the hammerhead ribozyme (Werner & Uhlenbeck, 1995).

In the DNA cleavage reaction with substrates that form single-base mismatches in either the P1 or P10 helix, catalytic efficiency was reduced due to deleterious effects on both k_{cat} and K_m. Unlike the situation with the U5 LTR_{P1} ribozyme, the U5 LTR_{P1+P10} ribozyme forms a highly stable IGS•substrate duplex. Thus, a duplex destabilization model cannot explain the high specificity of the P1+P10 ribozyme because a single mismatch is unlikely to cause the rate of duplex dissociation to be faster than the rate of duplex docking into the catalytic core. Rather, the effect of a mismatch may be to distort the IGS•substrate duplex in a way that impairs the docking process or subsequent positioning of the substrate within the catalytic core. Mismatches that occur closer to the cleavage site are expected to produce greater distortion of the proper geometry compared to those that lie farther away and thus have a more pronounced effect on catalytic activity.

A notable exception to the distance-from-cleavage-site rule involves mismatches at the +1 position. In the case of the U5 LTR_{P1+P10} ribozyme, a rU•dT or rU•dC mismatch at this position reduced activity 350- or 200-fold, respectively. In contrast, almost no activity was detected when either a rC•dT or rC•dA mismatch was introduced at the +2 position, even though this position is more distant from the cleavage site. During the exon ligation step of the self-splicing reaction catalyzed by the wild-type *Tetrahymena* ribozyme, a U•U mismatch is present at the +1 position. The DNA cleavage reaction catalyzed by the evolved ribozyme is analogous to the reverse of exon ligation, which may account for the ability of the ribozyme to tolerate mismatches at this position.

Our analysis has provided some insight into the ability of an *in vitro*-evolved ribozyme to recognize and cleave single-stranded DNA substrates in a sequence-specific manner. Among the ribozymes that were investigated, one that targeted a 12-nucleotide site within the U5 LTR region of HIV-1 cDNA had the highest level of catalytic activity and was studied in the greatest detail. This ribozyme, or one of its evolutionary descendants, might be considered as a potential therapeutic agent. The U5 LTR target sequence is highly conserved among known isolates of HIV-1 (Yu et al., 1993) and is a long-lived intermediate that is produced at the outset of the viral replication cycle. However, further studies must determine whether this or other target sequences will be accessible in the context of the viral cDNA and whether the cDNA itself is accessible to a ribozyme *in vivo*.

ACKNOWLEDGMENT

We thank Matthew Anderson and Hyonju Chong for technical assistance, Joyce Tsang for providing the evolved DNA-cleaving ribozyme, Luc Jaeger for the preparation of Figure 1b, and Dan Herschlag for helpful comments on the manuscript.

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BI960845G