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Comparative Study of DNA Enzymes and Ribozymes against the Same Full-length Messenger RNA of the Vanilloid Receptor Subtype I*

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The efficiencies of 32 antisense oligodeoxynucleotides, 35 DNA enzymes and 6 ribozymes to bind and cleave the full-length messenger RNA of the vanilloid receptor subtype I were analyzed. Systematic screening of the mRNA revealed that good accessibility of a putative cleavage site for antisense oligodeoxynucleotides is a necessary but not a sufficient prerequisite for efficient DNA enzymes. Comparison of DNA enzymes and ribozymes against the same target sites revealed: 1) DNA enzymes were more active with longer recognition arms (9 nucleotides on either side), whereas ribozymes revealed higher activities with shorter recognition arms (7 nucleotides on either side). 2) It does not only depend on the target site but also on the enzyme sequence, whether a DNA enzyme or a ribozyme is more active. 3) The most efficient DNA enzyme found in this study had an ~15-fold higher reaction rate, k_{react}, and a 100-fold higher k_{react}/K_m under single turnover conditions compared with the fastest ribozyme. DNA enzymes as well as ribozymes showed significant activity under multiple turnover conditions, the DNA enzymes again being more active. We therefore conclude that DNA enzymes are an inexpensive, very stable and active alternative to ribozymes for the specific cleavage of long RNA molecules.

DNA enzymes and ribozymes are catalytic nucleic acids that recognize and cleave a target RNA in a highly sequence-specific manner. They can therefore be used as therapeutic agents to inhibit the expression of deleterious genes. DNA enzymes and ribozymes bind to the target RNA by Watson-Crick base pairing and subsequently catalyze the cleavage of a phosphodiester bond.

The hammerhead ribozyme has been known for many years and is therefore well characterized. It consists of two hybridizing arms, which are complementary to the target RNA, a catalytic core and a helix. The crystal structure of the hammerhead ribozyme has been solved and revealed a Y-shaped form of the enzyme-substrate complex (1–3). Hammerhead ribozymes are suitable for specific down-regulation of gene expression, because they can cleave any RNA containing a NUH triplet (N: any nucleotide; H: A, C, or U), the sequence GUC being cleaved most efficiently.

Ribozymes have been used for specific gene inhibition to overcome drug resistance, cancer growth, viral diseases, and arthritis (for reviews, see Refs. 4–8). For investigations in cell culture or in animal model systems the ribozymes can be delivered in two ways: exogenous delivery of preformed ribozymes or endogenous delivery by transfection of the cells with the ribozyme genes (gene therapy). Exogenously delivered ribozymes have to be stabilized by introduction of modified nucleotides (*e.g.* 2′-fluoro, 2′-NH_2, or 2′-*O*-methyl modifications). For the endogenous delivery the choice of the promotor is important.

RNA-cleaving DNA enzymes have been isolated by *in vitro* selection using a combinatorial DNA library (9). The most efficient DNA enzyme, named "10-23" consists of two hybridizing arms and a highly conserved catalytic core of 15 nucleotides. It can cleave any junction between a purine and a pyrimidine. Catalytic efficiency of DNA enzymes is comparable with that of ribozymes. A crystal structure of the DNA enzyme in the catalytically active conformation is not known yet.

In a growing number of reports DNA enzymes are used to inhibit gene expression in various cell types (for reviews, see Refs. 7 and 8). Targets comprise virus RNAs (9–12) as well as mRNAs of oncogenes (10, 13), receptors (14), and further deleterious genes like BCR-ABL fusion (15–17) and Huntingtin (18). A DNA enzyme against the early growth response factor-1 (Egr-1) was used in an animal model. It could inhibit neointima formation after balloon injury to the rat carotid artery wall (19).

However, systematic studies comparing ribozymes and DNA enzymes against the same full-length target mRNA are rare. In many cases only one type of RNA-cleaving nucleic acid was used and very often kinetic experiments were only performed with short synthetic substrates. Our aim was to develop a strategy to find optimal DNA enzymes and ribozymes against the same mRNA target and to compare their activities.

Thus, we chose the mRNA of the vanilloid receptor subtype 1 (VR1),[1] also known as the Capsaicin receptor, as a target for the development of DNA enzymes and hammerhead ribozymes. The receptor is a cation channel, which is predominantly expressed by primary sensory neurons (20). It can be activated by capsaicin, the pungent component of hot chili peppers, protons, and heat (>43 °C). Therefore, the Capsaicin receptor is thought to be a new target for pain therapy. VR1 knockout mice did not develop thermal hyperalgesia after inflammation (21, 22).

The first step for DNA enzyme and ribozyme strategies is to identify accessible sites of the mRNA for the binding of oligonucleotides. We therefore characterized the VR1 mRNA (2614 nt) in an RNase H assay with 32 antisense oligodeoxynucleo-

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[1] The abbreviations used are: VR1, vanilloid receptor subtype 1; HPV, human papilloma virus; nt, nucleotides; ODN, oligodeoxynucleotide; VR1L1, vanilloid receptor type 1 like protein 1.

tides (ODN) against all putative GUC cleavage sites for DNA enzymes and ribozymes. Subsequent systematic screening of DNA enzymes revealed that they can only cleave sites which are accessible for the antisense ODNs. Ribozymes were directed against sites with high accessibility for antisense ODNs. The influences of the number of nucleotides in the substrate recognition arms on the activity of DNA enzymes and ribozymes were also investigated. Kinetic analysis showed that the most efficient DNA enzyme has a 15-fold higher reaction rate under single turnover conditions compared with the fastest ribozyme.

EXPERIMENTAL PROCEDURES

In Vitro Transcription of Substrate RNA—Rat VR1 was cloned from total RNA prepared from L4-L6 dorsal root ganglia taken from adult rats. Total RNA (2.5 μg) was reverse transcribed using oligo(dT) and Superscript reverse transcriptase (Invitrogen, Paisley, UK). Subsequent polymerase chain reaction was carried out with specific VR1 forward and reverse primers designed using the GenBank™ sequence AF029310. Primers corresponding to nucleotides 73–91 and 2578–2597 were rVR1F-*Eco*RI (5′-GCGCGAATTCTGGAAAGGATGGAACAACG-3′) and rVR1-*Xba*I (5′-GCGCTCTAGATTATTTCTCCCCTGGGACC-3′). *Eco*RI and *Xba*I restriction sites were introduced as indicated. A 2.5-kb fragment was amplified using *Pfu* DNA Polymerase (Stratagene, La Jolla, CA) and cloned into the *Eco*RI-*Xba*I sites of pcDNA3.1(+) (Invitrogen, Groningen, The Netherlands).

Sequence analysis revealed three single nucleotide differences compared with the sequence published by Caterina *et al.* (20). One of the mutations transforms a GUC triplet into GUU. The deviations were found in several clones and are also published in GenBank™ as vanilloid receptor type 1 like protein 1 (VR1L1) by Tsutsumi *et al.* (accession number AB040873). The differences might either be due to single nucleotide polymorphisms or sequencing errors of the originally published clone. The plasmid was linearized with *Xba*I before *in vitro* transcription with T7 RNA polymerase, which was performed with the RiboMAX Large Scale RNA Production System from Promega (Madison, WI).

Ribozymes and DNA Enzymes—Antisense oligodeoxynucleotides and DNA enzymes were obtained from MWG Biotech AG, Ebersberg, Germany. Ribozymes were synthesized by solid-phase chemistry on a PCR-MATE EP model 391 DNA synthesizer (Applied Biosystems) in a 1-μmol scale with standard phosphoramidites from Proligo Biochemie, Hamburg, Germany. Oligonucleotides were synthesized following standard procedures and purified by high performance liquid chromatography. Large amounts of ribozyme 16 (7/7) were obtained from IBA-NAPS Göttingen, Germany.

Messenger Walk Screening—Messenger walk screening was performed to identify accessible sites for antisense oligodeoxynucleotides. Therefore, 100 nM VR1 mRNA were incubated with a 5-fold excess of an antisense oligodeoxynucleotide in a total volume of 10 μl in 40 mM Tris/HCl, pH 7.2, 4 mM $MgCl_2$, 1 mM dithiothreitol, and 150 mM NaCl for 7.5 min at 37 °C in the presence of 0.4 units of RNase H (Promega, Madison, WI). The reaction was stopped by addition of EDTA (final concentration: 83 mM). Uncleaved substrate and digestion products were separated on a 1.5% agarose gel and stained with ethidium bromide. Agarose gels were preferred instead of polyacrylamide gels due to the well known difficulties to separate long RNAs (>2000 bases) with the latter. The signals of ethidium bromide-stained RNAs were found to be linear with the concentration and the ratio of uncleaved substrate and digestion products was independent from the signal intensity. The gels were photographed with the Gel Doc 2000 Gel Documentation System and quantitatively evaluated with the program Quantity One (Bio-Rad, Munich, Germany). All values given are the average and standard deviation of at least three independent experiments.

Ribozyme and DNA Enzyme Kinetics—Kinetics of DNA enzymes and ribozymes were investigated under single and multiple turnover conditions. Experiments were performed in 50 mM Tris/HCl, pH 7.5, and 10 mM $MgCl_2$ at 37 °C. 1.25 units/μl RNasin were added to prevent degradation of the mRNA. Ribozymes and DNA enzymes were denatured at 65 °C for 3 min and subsequently cooled down to 37 °C. Reactions were started by addition of the enzymes to the substrate mRNA (final concentration: 100 nM). The ribozymes and DNA enzymes were used to give a final concentration of 1, 5, and 10 μM for single turnover experiments and 10 nM for multiple turnover experiments. Aliquots were removed at different time points and the reactions were stopped by the addition of 83 mM EDTA and subsequent snap-cooling on ice. The cleavage reactions were analyzed on agarose gels as described above.

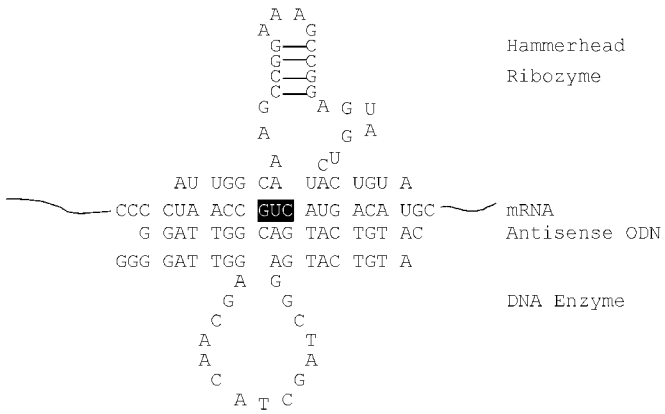


FIG. 1. **Part of the VR1 sequence with the GUC cleavage site 15 in the translated region (*black box*).** Sequences of an 18-mer antisense oligodeoxynucleotide and DNA enzyme with 9-nt recognition arms are given *below* and the sequence of the hammerhead ribozyme with 7-nt recognition arms is given *above* the mRNA.

For single turnover experiments the time-dependent decrease of the uncleaved fraction was fitted with a monoexponential decay function by Origin (Microcal Software, Northampton, MA). Kinetics for DNA enzyme 15 (9/9) under single turnover conditions could only be fitted with a biexponential decay function. Further kinetic evaluation of the data followed standard procedures described in the literature (23, 24) with some modifications, since an exhaustive kinetic characterization is impossible for long substrate RNA molecules like full-length mRNAs due to limitations of material. Therefore, only experiments with 10-, 50-, and 100-fold enzyme excess were performed and reaction rates, k_{obs}, were determined. All k_{obs} values used are the average including standard deviation of three independent experiments. Maximal reaction rates, k_{react}, and K_m values were estimated by hyperbolic fitting of k_{obs} values, which were plotted as a function of the enzyme concentration. Again, all values are the average including standard deviation of three independent sets of experiments.

For multiple turnover experiments the initial reaction velocity was calculated by linear fitting of data points obtained in the first 15 min of the experiment. All values given are the average including standard deviation of at least three independent experiments.

RESULTS

Site Selection—The first step for the development of ribozymes and DNA enzymes is the selection of a suitable cleavage site. GUC triplets in the target RNA are most efficiently cleaved by hammerhead ribozymes, and since any junction of a purin and a pyrimidin can be cleaved by a 10-23 DNA enzyme, the GU(C) sequences can also be used as target site for DNA enzymes. Therefore, all 32 GUC triplets in the translated region of the VR1 mRNA were analyzed as putative target sites. Messenger walk screening with antisense ODN complementary to the GUC triplets was used to identify sites of the mRNA, which are accessible to oligonucleotides. The antisense ODNs were 18-mers with a GAC triplet in the center and the general sequence: NNNNNNNNGACNNNNNNN. As an example, antisense ODN against GUC site 15 is shown in Fig. 1. A 5-fold excess of the antisense ODNs was added to the mRNA in the presence of RNase H, which cleaves DNA/RNA duplexes that were formed, wherever an ODN can bind to the mRNA.

Fig. 2 (*black bars*) shows that mRNA cleavage of more than 90% could be achieved with the most efficient antisense ODN (number 29) in the presence of RNase H under conditions described above. Five further antisense ODNs (numbers 2, 15, 16, 25, and 27) led to a cleavage of more than 70% of the mRNA. The sequences of the two most efficient antisense ODNs are: ODN number 15, CATGTCATGACGGTTAGG and number 29, ATCTTGTTGACGGTCTCA.

DNA enzymes were designed against the 32 GUC triplets analyzed by messenger walk screening. The general sequence

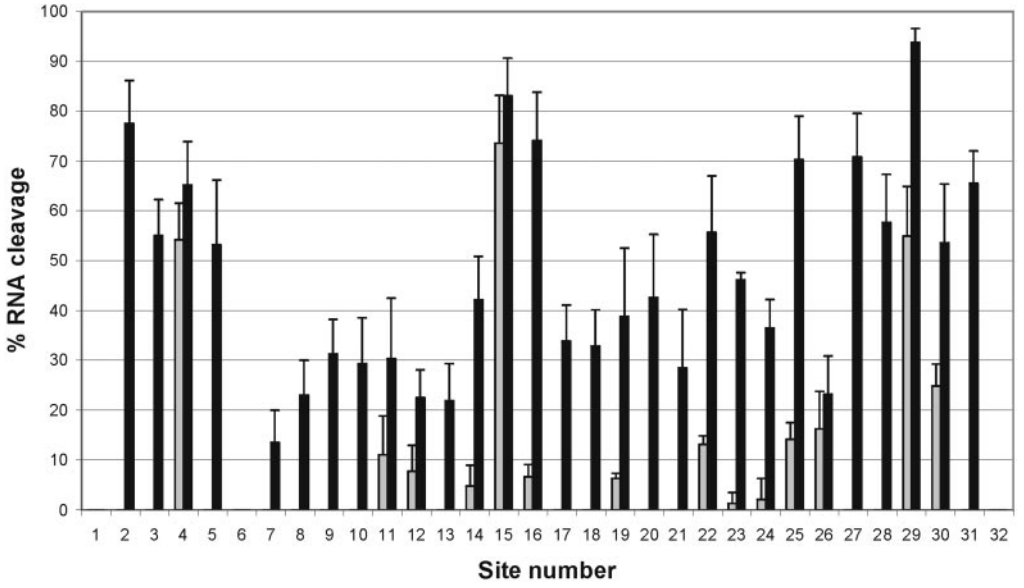


FIG. 2. **Messenger walk and DNA enzyme screening for VR1 mRNA.** The fraction of cleaved mRNA by RNase H is shown for each of the 32 antisense oligonucleotides directed against all putative hammerhead cleavage sites GUC (*black bars*). The antisense ODNs were added to the mRNA in 5-fold excess, followed by a 7.5-min incubation at 37 °C. Cleavage of the mRNA by DNA enzymes (10-fold excess to the mRNA) against the same sites after 20 min incubation at 37 °C is show with *gray bars*. All values are averages of at least three independent experiments.

for a 10-23 DNA enzyme with two recognition arms of nine nucleotides on either side is: NNNNNNNNNGGCTAGCTACAACGANNNNNNNNN. The DNA enzyme against GUC site number 15 is shown in Fig. 1.

Cleavage of mRNA by DNA enzymes under single turnover conditions (10-fold excess of DNA enzymes over the substrate RNA) after 20 min incubation at 37 °C is shown in Fig. 2 (*gray bars*). All DNA enzymes were denatured prior to the assay. More than half of the DNA enzymes were completely inactive and only four DNA enzymes (13%) cleaved more than 20% of the mRNA.

A comparison of the activity of the DNA enzymes with the results obtained by messenger walk screening revealed that highly efficient DNA enzymes were only obtained for sites which are well accessible to antisense ODNs. However, accessibility of the site is only a necessary but not a sufficient prerequisite for good DNA enzymes. Many DNA enzymes were totally inactive even though the site was accessible for oligonucleotides. Obviously, the accessibility of a site is not the only crucial factor for the activity of a DNA enzyme. The formation of internal secondary structures of the enzyme may lead to inactive conformations.

Comparison of DNA Enzymes and Ribozymes under Single Turnover Conditions—Due to the relative expensive RNA synthesis, we refrained from a systematic screening of ribozymes against all GUC sites. Therefore, hammerhead ribozymes against three of the sites with highest accessibility for oligonucleotides (numbers 15, 16, and 29) in the messenger walk screening were synthesized. The general sequence of a hammerhead ribozyme against a GUC site with 7-nt antisense arms is: NNNNNNNCUGAUGAGGCCGAAAGGCCGAAACNNNNN. The hammerhead ribozyme cleaving GUC site number 15 is shown in Fig. 1.

Results for ribozymes and DNA enzymes against site numbers 15, 16, and 29 are shown in Fig. 3 (top). To analyze the influence of the length of the substrate recognition arms on the cleavage activity, ribozymes and DNA enzymes with 7 and 9 nucleotides in each binding arm were compared.

Quantitative evaluation of the gel (Fig. 3, *bottom*) reveals three interesting features. 1) DNA enzymes are more efficient with longer antisense arms whereas ribozymes have a higher activity with shorter arms. 2) It does not only depend on the target site, but also on the enzyme sequence, whether a ribozyme or a DNA enzyme is more active. For sites 15 and 29 DNA enzymes had a higher cleavage activity, whereas the ribozyme against site number 16 was more efficient than the DNA enzyme against the same target site. 3) The most efficient DNA enzyme found cleaves the full-length target mRNA more efficiently than the most active ribozyme.

To further characterize the DNA enzymes and ribozymes with high cleavage activity kinetic experiments under single turnover conditions were performed. The sequences of these ribozymes and DNA enzymes, which were named after the sites they are targeted against and the length of the substrate recognition arms in parentheses, are: Ribozyme 15 (7/7): AUGUCAUCUGAUGAGGCCGAAAGGCCGAAACGGUUA; Ribozyme 16 (7/7): UGCGCUUCUGAUGAGGCCGAAAGGCCGAAACAAAUC; DNA enzyme 15 (9/9): ATGTCATGAGGCTAGCTACAACGAGGTTAGGGG; and DNA enzyme 29 (9/9): TCTTGTTGAGGCTAGCTACAACGAGGTCTCACC.

Fig. 4 (*top*) is an example of mRNA cleavage by a 100-fold excess of DNA enzyme 15 (9/9) at different time points. This DNA enzyme cuts the substrate (S) into two product fragments (P1 and P2) of equal length, and therefore only one product band is observed. In Fig. 4 (*bottom*) the fraction of uncleaved mRNA in the presence of DNA enzymes and ribozymes (100-fold excess) is shown as a function of time. The kinetics of mRNA cleavage for the DNA enzyme 15 (9/9) are biphasic. Data for all other enzymes can be described by monoexponential decay kinetics. The observed cleavage rates and corresponding amplitudes at 100-fold enzyme excess are summarized in Table I. For the most active DNA, enzyme 15 (9/9), approximately half of the reaction takes place with a fast phase of 2.8 min^{-1}. Interestingly, for DNA enzyme 29 (9/9) which is slower than DNA enzyme 15 (9/9) and ribozyme 16 (7/7) the smallest fraction of substrate mRNA remains uncleaved when the reaction reaches completion.

Due to limitations of material an exhaustive kinetic characterization is not possible for the cleavage of long substrate molecules. The kinetic characterization method frequently used for long substrate molecule, in which only one time point is measured for each enzyme concentration (24) could also not

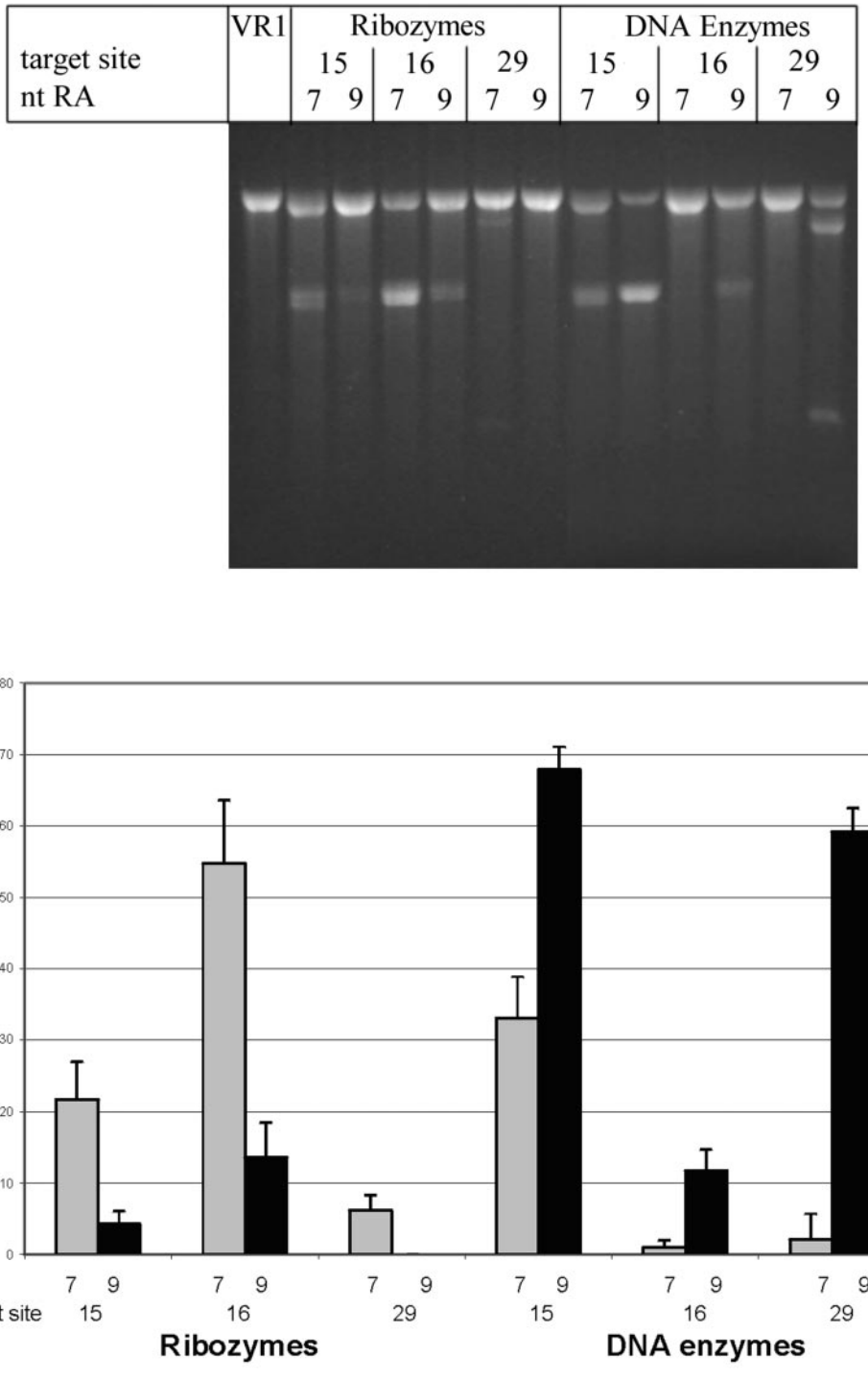


FIG. 3. **VR1 mRNA cleavage by ribozymes and DNA enzymes against sites 15, 16, and 29 under single turnover conditions (10-fold enzyme excess, 20 min at 37 °C).** The length of the recognition arms (*RA*) was 7 and 9 nucleotides as indicated in the figure. *Top*, ethidium bromide-stained agarose gel. *Bottom*, quantitative evaluation of the gel. All values are averages of at least three independent experiments.

be applied because of the biphasic behavior of DNA enzyme 15 (9/9). We therefore determined k_{obs} for different enzyme excess (10-, 50-, and 100-fold) as described above and plotted the observed cleavage rate k_{obs} against the enzyme concentration. Hyperbolic fits were used to estimate kinetic parameters, which are summarized in Table II. The maximal reaction rate for the most active DNA enzyme is ~15-fold higher than the rate for the most efficient ribozyme 16 (7/7). K_m values are lower for DNA enzymes than for ribozymes. As a consequence k_{react}/k_M for DNA enzyme 15 (9/9) is ~100-fold higher than for ribozyme 15 (7/7).

Comparison of DNA Enzymes and Ribozymes under Multiple Turnover Conditions—The DNA enzymes and ribozymes were further characterized under multiple turnover conditions, *i.e.* with an enzyme to substrate ratio of 1:10. Ribozymes against site 29 and DNA enzymes against site 16 did not have significant cleavage activity under these conditions (data not shown). Fig. 5 (*top*) shows the results of mRNA cleavage by the remaining enzymes after 2 h at 37 °C. Quantitative evaluation of the gel (Fig. 5, *bottom*) confirms the results, which were already obtained under single turnover conditions: 1) DNA enzymes are more efficient with substrate recognition arms of 9 nucle-

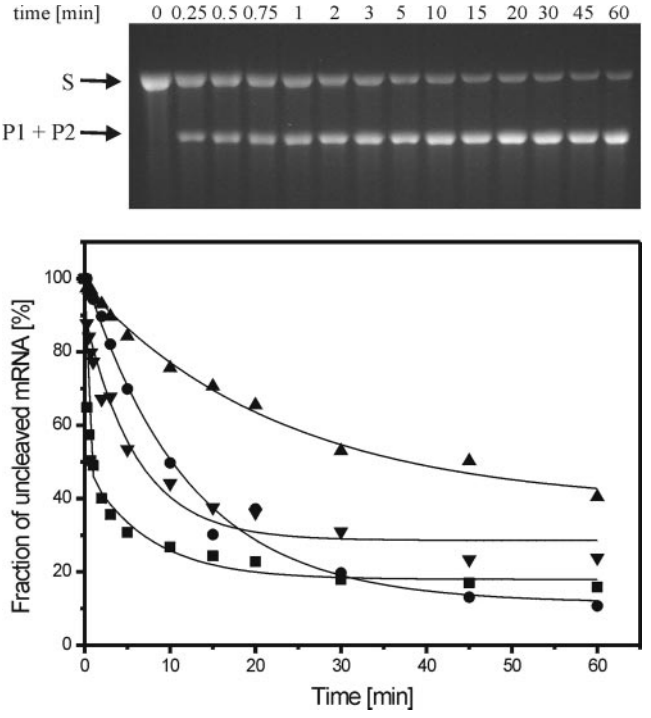


FIG. 4. *Top*, cleavage of the full-length VR1 mRNA by DNA enzyme 15 (9/9) under single turnover conditions. The gel shows electrophoretically separated digestion products (*P1* and *P2*) derived from the full-length mRNA substrate (*S*) after incubation at 37 °C for various time points ranging from 0 to 60 min. *Bottom*, kinetics of cleavage of full-length VR1 mRNA under single turnover conditions by ribozymes 15 (7/7) (▲) and 16 (7/7) (▼) and DNA enzymes 15 (9/9) (■) and 29 (9/9) (●). Enzymes were added at 100-fold excess to the mRNA followed by incubation at 37 °C. At appropriate times, aliquots were removed from the reaction. Substrate and digestion products were separated electrophoretically and the ethidium bromide-stained gel was quantitatively evaluated to give the fraction of uncleaved mRNA at each time point.

otides whereas ribozymes have a higher activity with shorter arms. 2) The fastest DNA enzyme cleaves the VR1 mRNA more efficiently than the most active ribozyme.

It is well known that it is difficult to observe the cleavage of long target RNAs under multiple turnover conditions (25). However, for ribozyme 16 (7/7) and DNA enzyme 15 (9/9) significant multiple turnover activity with the full-length mRNA could be detected (Fig. 6).

The initial reaction velocity was calculated by linear fitting of data points obtained in the first 15 min of the experiment (Table III). Values of 0.29 ± 0.07 nM/min and 0.8 ± 0.1 nM/min were obtained for ribozyme 16 (7/7) and DNA enzyme 15 (9/9), respectively, *i.e.* the initial reaction velocity at 10-fold substrate excess is 2.8-fold higher for the DNA enzyme.

DISCUSSION

The aim of the present study was the development of a strategy for the design of highly efficient ribozymes and DNA enzymes for the cleavage of long RNA transcripts and comparison of their activities against the same sites in the full-length target mRNA of the vanilloid receptor subtype 1. We first characterized the structure of the mRNA by messenger walk screening. For this purpose, antisense oligodeoxynucleotides against all 32 GUC triplets in the translated region, which are putative cleavage sites for hammerhead ribozymes, were added to the mRNA. RNase H should cleave the RNA at those positions at which an antisense ODN has been bound. Several antisense ODNs were found to mediate substantial cleavage of the mRNA by RNase H. These oligodeoxynucleotides can now be used to investigate pain perception in animal models. In a recent study, carrageenan treatment was shown to induce axonal transport of the VR1 mRNA from the dorsal root ganglia to central and peripheral axon terminals (26). The level of VR1 mRNA in the lumbar dorsal horn could be almost abolished by intrathecal injection of an antisense ODN complementary to the first 20 nucleotides of the translated region.

DNA enzymes which cleave any purin/pyrimidin junction were also directed against the 32 GU(C) sites within the translated region of the VR1 mRNA. Comparison of their ability to digest the mRNA with the antisense ODN-mediated mRNA cleavage by RNase H revealed that good accessibility of a putative cleavage site for an antisense ODN is an essential but not sufficient prerequisite for an efficient DNA enzyme, since some DNA enzymes were completely inactive although the antisense ODN could bind to the mRNA. This result was confirmed for other target mRNAs as well.[2] The finding, that more than half of the DNA enzymes tested in this study were inactive, is also in agreement with results from a systematic screening of 80 DNA enzymes against the HPV16 E6 transcript, where even 90% of the deoxyribozymes did not show substantial cleavage activity (10).

An analogous comparison of the accessibility of sites in a messenger RNA for antisense oligodeoxynucleotides and their cleavage by hammerhead ribozymes was performed for the proto-oncogene c-*myb* (27). The results obtained for hammerhead ribozymes were similar to the finding of the present study for DNA enzymes. In general, the sites that were accessible to antisense oligonucleotides were also susceptible for cleavage by ribozymes. However, some exceptions were found, accessible sites for antisense ODNs which were not cleaved by ribozymes as well as efficiently cleaved sites which were poorly accessible in the RNase H assay. In addition to the accessibility of a cleavage site, formation of internal secondary structures, steric hindrance due to the catalytic center, inability of formation of the active conformation, and thermodynamics of enzyme-substrate interactions might be important factors for the catalytic activity of DNA enzymes and ribozymes.

The accessibility of cleavage sites for hammerhead ribozymes was further investigated by chemical modification mapping of the HIV-1 *vif-vpr* transcript (28). These experiments revealed that availability of nucleotides close to the cleavage site for base pairing with the ribozyme is important for efficient cleavage of a long RNA, whereas steric hindrance from target RNA structures is unlikely to affect hammerhead ribozyme cleavage.

A variety of strategies has previously been used to screen RNA targets for accessible sites (for a review, see Ref. 29). An oligonucleotide array was designed to map an RNA for hybridization sites for antisense oligodeoxynucleotides (30). An alternative approach was the use of random or semirandom ODN libraries and RNase H followed by primer extension (31, 32). In a nonrandom variation of this strategy target-specific oligonucleotides were generated by digestion of the template DNA (33). All of these methods are labor intensive and expensive due to the primer extension analysis and do not reveal further information to the rather simple messenger walk screening presented in this study. Therefore, this method seems to be a fast, cheap, and easy alternative way to identify suitable cleavage sites for DNA enzymes and ribozymes.

Since DNA enzymes and ribozymes can still be completely inactive even if the binding region of the mRNA is accessible for antisense ODNs site selection with the catalytic nucleic acids themselves might be advantageous. Efficient cleavage sites for ribozymes were found by the use of libraries with randomized

[2] J. Kurreck, B. Bieber, S. Schubert, and V. Erdmann, unpublished data.

TABLE I
Cleavage rates and corresponding amplitudes of VR1 mRNA cleavage at 100-fold enzyme excess

	A_1	k_1	A_2	k_2	A_∞
	%	min^{-1}	%	min^{-1}	%
DNAzym 15 (9/9)	48 ± 3	2.8 ± 0.9	30 ± 2	0.13 ± 0.01	21 ± 5
DNAzym 29 (9/9)	89 ± 1	0.084 ± 0.006			11 ± 0.4
Ribozym 15 (7/7)	66 ± 11	0.041 ± 0.006			33 ± 11
Ribozym 16 (7/7)	61 ± 1	0.17 ± 0.03			29 ± 2

TABLE II
Estimated kinetic parameters of VR1 mRNA cleavage under single turnover conditions

	k_{react}	K_M	k_{react}/K_M
	min^{-1}	nM	min^{-1} μM^{-1}
DNA enzyme 15 (9/9)	2.7 ± 0.9	210 ± 40	13 ± 7
DNA enzyme 29 (9/9)	0.092 ± 0.009	640 ± 20	0.14 ± 0.02
Ribozyme 15 (7/7)	0.042 ± 0.006	1000 ± 300	0.04 ± 0.02
Ribozyme 16 (7/7)	0.18 ± 0.04	1500 ± 300	0.12 ± 0.05

(34, 35) or sequence-specific (36) substrate recognition arms. Highly active DNA enzymes against the HIV gag transcript were isolated from combinatorial libraries of randomized or partially randomized DNA enzymes (37). To find the optimal DNA enzyme against the human papilloma virus HPV 16 E6 transcript the cleavage pattern of a mixture of 80 specific deoxyribozymes was analyzed by primer extension (10). Again, these methods are more labor intensive than the screening with single DNA enzymes we performed and the best DNA enzyme found for the HPV RNA was less active than the fastest DNA enzyme identified by our method (see below).

Due to high costs, the activity of hammerhead ribozymes were not screened systematically for all putative cleavage sites. Therefore, we only designed ribozymes to cleave three of the sites with highest accessibility for antisense ODNs. Interestingly, ribozymes were more active with substrate recognition arms of 7 nucleotides on either side, whereas DNA enzymes cleaved the mRNA more efficiently with longer arms of 9 nucleotides on either side. This result is in agreement with findings for hammerhead ribozymes against the c-*myb* mRNA (27). Symmetric ribozymes with arm lengths ranging from 5 to 12 nucleotides were tested and the 7/7-nt arm ribozymes were found to be most efficient. However, it cannot be generalized that ribozymes are more active with shorter arms. Hammerhead ribozymes against the full-length interleukin-2 RNA were more effective with longer antisense arms although a short synthetic substrate could be cleaved with highest efficiency by a ribozyme with short recognition arms (38). A computer predicted secondary structure of the long interleukin-2 target RNA revealed a single stranded loop region in the vicinity to the cleavage site, which can only be used for hybridization by ribozymes with longer recognition arms. In the case of DNA enzymes, our results are in agreement with a study demonstrating that deoxyribozymes cleaved the full-length c-*myc* mRNA more efficiently with longer than with shorter antisense arms (13). However, no general conclusion can be drawn whether symmetric or asymmetric substrate recognition arms are superior. For a target sequence derived from HPV16 E7 a DNA enzyme with symmetric arms of 10 nucleotides on each side showed the highest activity, whereas DNA enzymes with asymmetric arms were more efficient to cleave a c-*myc* target sequence (39).

Under multiple turnover conditions identical results were obtained for the variation of the arm length as under single turnover conditions. Ribozymes were more efficient with 7 nucleotides and DNA enzymes with 9 nucleotides on either side. Long substrate recognition arms can drastically reduce activities of ribozymes due to slow product release. However, the DNA enzymes used in this study were more active with longer than with shorter antisense arms even under multiple turnover conditions. In contrast to our results obtained with a long RNA transcript as a target, k_{cat} was found to be similar for substrate recognition domains from 7 to 9 nucleotides on either side of DNA enzymes against short RNA targets (40).

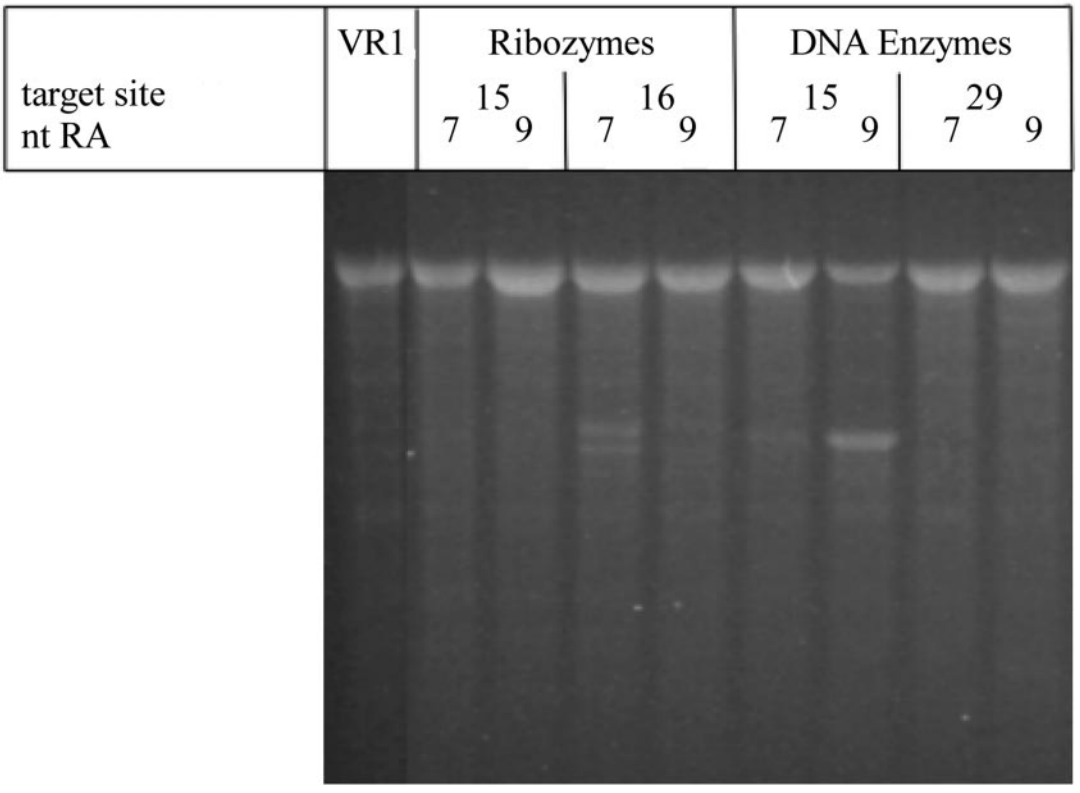
To our knowledge, our study is the first comparison of DNA enzymes and ribozymes not only against the same long RNA, but also against the same target sites within the mRNA. Our results show, that it is not possible to draw a general conclusion, whether the hammerhead ribozyme or the DNA enzyme has a higher activity. In two cases (sites 15 and 29) the DNA enzyme was superior and in one case (site 16) the hammerhead ribozyme was more efficient. Cleavage activity obviously not only depends on the accessibility of the target site, but also on the sequence of the catalytic nucleic acid and the formation of favorable internal structures.

Kuwabara *et al.* (15) directed hammerhead ribozymes and DNA enzymes against the mRNA of the BCR-ABL fusion gene, which causes chronic myelongenous leukemia, and found the ribozyme being more active than the DNA enzymes. In contrast, Goila and Banerjea (14) found a DNA enzyme being more effective in cleaving the chemokinin receptor CCR5 transcript than a hammerhead ribozyme. However, in both studies, the DNA enzymes and ribozymes were directed against different target sites and their activities can therefore not be compared. To be able to make a comparison we used the same target sites for both kinds of RNA cleaving nucleic acids and found that either the DNA enzyme or the hammerhead ribozyme may be superior.

Still, the study of Kuwabara *et al.* (15) demonstrated another advantage of the DNA enzymes. Although they found the ribozyme to be more active *in vitro* it could not be used for therapeutic purpose. Due to the lack of suitable hammerhead cleavage sites close to the BCR-ABL junction it cannot distinguish between the abnormal BCR-ABL mRNA and the normal ABL mRNA. In contrast, DNA enzymes which have less restrictions with regard to the cleavage site could be directed against the junction and cleavage occurred therefore only within the chimeric BCR-ABL junction.

In a number of studies, kinetic data for DNA enzymes and ribozymes were obtained for short substrates only. Hammerhead ribozymes are known to have cleavage rates of ~1 min^{-1} (23), the catalytic activity of DNA enzymes being comparable (9). The rates for the cleavage of long transcripts by ribozymes and DNA enzymes are only rarely determined, but it is well known that they are usually several orders of magnitude lower compared with the rates for the cleavage of short substrates. We therefore measured and compared kinetics for DNA enzymes and ribozymes against the VR1 transcript of 2614 nucleotides under single and multiple turnover conditions.

The first interesting finding was that DNA enzymes showed monophasic as well as biphasic cleavage kinetics. It was suggested that a biphasic behavior of ribozymes arises when an alternate inactive conformation of the enzyme-substrate complex can be formed that is only slowly exchanged with the



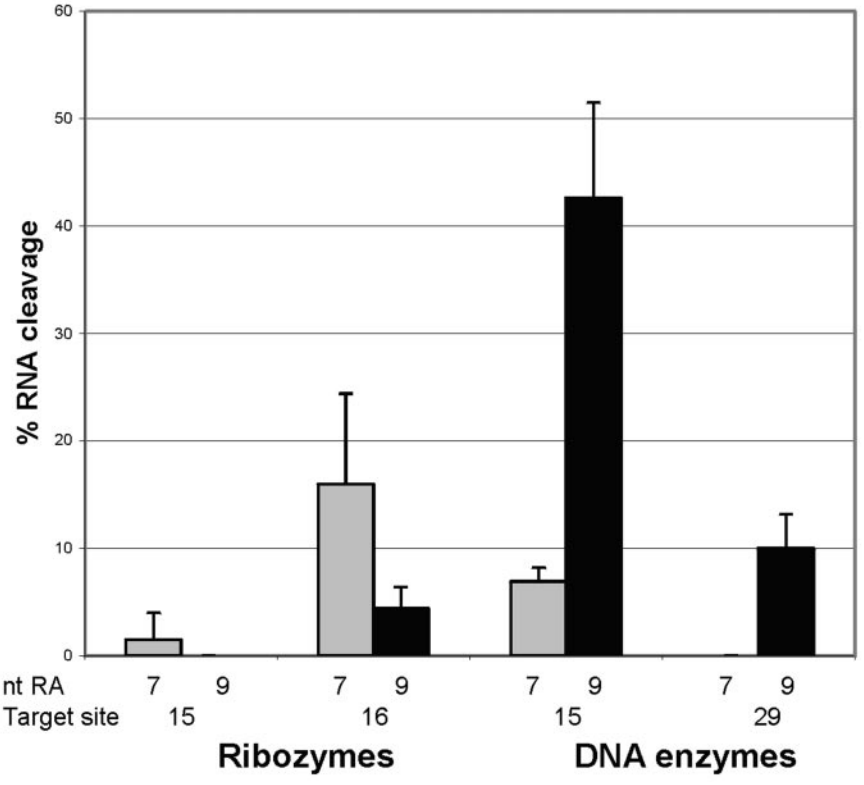


FIG. 5. **VR1 mRNA cleavage by ribozymes against sites 15 and 16 and by DNA enzymes against sites 15 and 29 under multiple turnover conditions (10-fold substrate excess, 2 h at 37 °C).** The length of the recognition arms (*RA*) was 7 and 9 nucleotides as indicated in the figure. *Top*, ethidium bromide-stained agarose gel. *Bottom*, quantitative evaluation of the gel. All values are averages of at least three independent experiments.

active conformation (23). A similar explanation can be drawn for DNA enzymes.

The second interesting finding was that the most efficient DNA enzyme 15 (9/9) had a 15-fold higher maximal reaction rate compared with the most effective hammerhead ribozyme. The rate of the DNA enzyme of 2.7 min^{-1} is even higher than

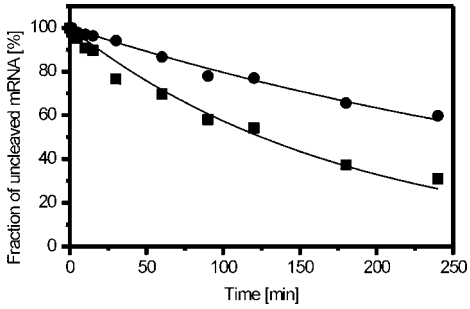


FIG. 6. **Cleavage of full-length VR1 mRNA under multiple turnover conditions by ribozyme 16 (7/7) (●) and DNA enzyme 15 (9/9) (■).** Enzymes were added to give a 10-fold excess of the mRNA followed by incubation at 37 °C. At appropriate times, aliquots were removed from the reaction. Substrate and digestion products were separated electrophoretically and the ethidium bromide-stained gel was quantitatively evaluated to give the fraction of uncleaved mRNA at each time point.

TABLE III
Initial velocity obtained under multiple turnover conditions

	v
	nM/min
DNA enzyme 15 (9/9)	0.8 ± 0.1
Ribozyme 16 (7/7)	0.29 ± 0.07

the k_{cat} of 0.49 min^{-1} obtained under comparable conditions for a DNA enzyme with a short substrate (9), demonstrating that there should be no hindrance due to the long transcript for the site attacked.

The higher K_m values which we obtained for ribozymes may be a consequence of the shorter substrate binding arms of seven nucleotides needed for maximal cleavage activity compared with nine nucleotides for DNA enzymes. Therefore, the target RNA is bound less tightly by ribozymes. As a consequence of the higher k_{react} and the lower K_m for the DNA enzyme 15 (9/9) the k_{react}/K_m is even ~100-fold higher than for the ribozyme 16 (7/7). Under multiple turnover conditions with 10-fold substrate excess the DNA enzyme still compares favorably with the ribozyme with a 2.8-fold higher initial reaction velocity.

Due to the problems with handling long RNAs and the lack of a standard method and standard conditions it is difficult to compare reaction rates obtained for DNA enzymes and ribozymes in different laboratories. Only a few examples can be used for comparison. The fastest DNA enzyme against the HPV E6 RNA found in a pool of 80 DNA enzymes had an observed rate of 0.21 min^{-1} (10), *i.e.* 1 order of magnitude slower than the fastest DNA enzyme (k_{react} = 2.7 min^{-1}) found in this study. Ribozymes described in the literature had even lower rates against long targets, *e.g.* Ribozyme 865 had a k_{react} of 0.0204 min^{-1} against luciferase mRNA (41) compared with a k_{react} of 0.18 min^{-1} for Ribozyme 16 (7/7) against the VR1 mRNA. Two interesting features arise from these data. 1) DNA enzymes compare favorably with hammerhead ribozymes. 2) The two highly active DNA enzymes were obtained by systematic screening of numerous DNA enzymes against the same long target RNA. In contrast to ribozymes oligodeoxynucleotides are relatively inexpensive and can be easily obtained. Therefore, it seems worth selecting the most efficient DNA enzyme out of a large number of candidates rather than just testing one DNA enzyme, *e.g.* against the start codon AUG.

One important point to be addressed is the question, whether DNA enzymes and ribozymes optimized under *in vitro* conditions are also efficient in cleaving the target RNA in cell culture and *in vivo*. The sites which were found to be accessible *in vitro* might be blocked under *in vivo* conditions by the formation of different secondary and tertiary structures or by proteins bound to the RNA. But several studies demonstrated that there is a correlation between the *in vitro* and intracellular activity. The *in vitro* efficiency found for hammerhead ribozymes against the interleukin-2 mRNA was confirmed in cell culture (38). Cairns *et al.* (10) screened DNA enzymes against the c-*myc* target and assessed the relationship between *in vitro* cleavage activity and gene suppression in cell culture. They found that efficient c-*myc* cleavers *in vitro* induced the most substantial suppression of smooth muscle cell proliferation, which provided an indication of the respective biological response. In contrast, poor c-*myc* cleavers had no effect on the level of the cell proliferation. *In vitro* optimized ribozymes and DNA enzymes should therefore be efficient in cell cultures and *in vivo*. However, Amarzguioui *et al.* (42) found only little predictive power of *in vitro* accessibility assays for ribozyme efficiency in cell culture. They preferred secondary structure prediction of the target RNA by the MFold program for ribozyme optimization.

CONCLUSION

We have characterized the VR1 mRNA by messenger walk screening, *i.e.* by systematic addition of antisense oligodeoxynucleotides and RNase H to the target RNA, and found accessible cleavage sites for hammerhead ribozymes and DNA enzymes. No general conclusion can be drawn about the predictability whether the hammerhead ribozyme or the DNA enzyme is superior for a certain target site. But a kinetic comparison of the two kinds of RNA cleaving nucleic acids revealed that the most efficient DNA enzyme had a 15-fold higher reaction rate and an ~100-fold higher k_{react}/K_m than the best ribozyme. Since DNA enzymes have less restrictions concerning the cleavage sites than ribozymes and can easily be synthesized, the best cleaver for a target can be selected by systematic screening. These advantages together with their applicability in animal models (19) demonstrate the potential of DNA enzymes as therapeutic agents. The best antisense oligodeoxynucleotides, ribozymes, and DNA enzymes identified in this *in vitro* study will now be tested in animal models with regard to a modulation of pain perception.

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