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# Characterization in Terms of the NUX Rule of G-inserted Mutant Hammerhead Ribozymes with High Level of Catalytic Power

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**Attempts using *in vitro* and *in vivo* selection procedures have been made to search for hammerhead ribozymes that have higher activities than the wild-type ribozyme and also to determine whether other sequences might be possible in the catalytic core of the hammerhead ribozyme. Active sequences selected in the past conformed broadly to the consensus core sequence except at A9, and no sequences were associated with higher activity than that of the hammerhead with the consensus core, an indication that the consensus sequence derived from viruses and virusoids is probably the optimal sequence [Vaish *et al.* (1997) Biochemistry 36, 6495-6501]. Recently, during construction of ribozyme expression vectors, we isolated a mutant hammerhead ribozyme, with an insertion of G between A9 and G10.1, that appeared to show significant activity [Kawasaki *et al.* (1996) *Nucleic Acids Res.* 24, 3010-3016; Kawasaki *et al.* (1998) *Nature* 393, 284-289]. We, therefore, characterized kinetic properties of the G-inserted mutant ribozymes in terms of the NUX rule. We demonstrate that the NUX rule is basically applicable to the G-inserted ribozymes and, more importantly, one type of G-inserted ribozyme was very active with $k_{cat}$ value of 6.4 $min^{-1}$ in 50 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ at 37°C.**

**Keywords:** Ribozyme; Hammerhead; Catalytic core; NUX rule; p300; Mutant ribozymes

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## Introduction

The hammerhead ribozyme is one of the smallest RNA enzymes (Alberts *et al.*, 1994; Birikh *et al.*, 1997; Doudna, 1998; Walter and Burke, 1998; Zhou and Taira, 1998; Carola and Eckstein, 1999; Gesteland *et al.*, 1999; Kim and Park, 2000; Lilley, 1999; Scott, 1999; Warashina *et al.*, 2000). Because of its small size and potential utility as antiviral agents, it has been extensively investigated in terms of the mechanism of its action (Dahm and Uhlenbeck, *et al.*, 1991; Dahm *et al.*, 1993; Pyle, 1993; Hertel *et al.*, 1994; Sawata *et al.*, 1995; Baidya and Uhlenbeck, 1997; Birikh *et al.*, 1997; Hertel *et al.*, 1997; Pontius *et al.*, 1997; Lott *et al.*, 1998; Stage-Zimmermann and Uhlenbeck, 1998; Zhou and Taira, 1998; Scott and Uhlenbeck, 1999; Wang *et al.*, 1999; Derrick *et al.*, 2000; Warashina *et al.*, 2000; Yoshinari and Taira, 2000) and possible applications *in vivo* (Sarver *et al.*, 1990; Altman, 1993; Homann *et al.*, 1993; Mulligan, 1993; Ohkawa *et al.*, 1993; Cameron and Jennings, 1994; Kiehntopf *et al.*, 1994; Marschall *et al.*, 1994; Sullivan, 1994; Christoffersen and Marr, 1995; Kiehntopf *et al.*, 1995; Sun *et al.*, 1995; Thompson *et al.*, 1995a; Thompson *et al.*, 1995b; Tuschl *et al.*, 1995; Eckstein and Lilly, 1996; Ferbeyre *et al.*, 1996; Kawasaki *et al.*, 1996; Koseki *et al.*, 1998; Kuwabara *et al.*, 1998a,b,1999; Tanabe *et al.*, 2000). It was first recognized as the sequence motif responsible for self-cleavage (cis action) in the satellite RNAs of certain viruses (Symons, 1989). The putative consensus sequence required for activity has three duplex stems and a conserved "core" of two non-helical segments, plus an unpaired nucleotide at the cleavage site (Fig. 1). The *trans*-acting hammerhead ribozyme consists of an antisense section (stem I and stem III) and a catalytic domain with a flanking stem/loop II section (Uhlenbeck, 1987; Haseloff and Gerlach, 1988). The stem II can be a common stem in the case of dimeric short ribozymes (Amontov and Taira, 1996; Amontov *et al.*, 1996; Kuwabara

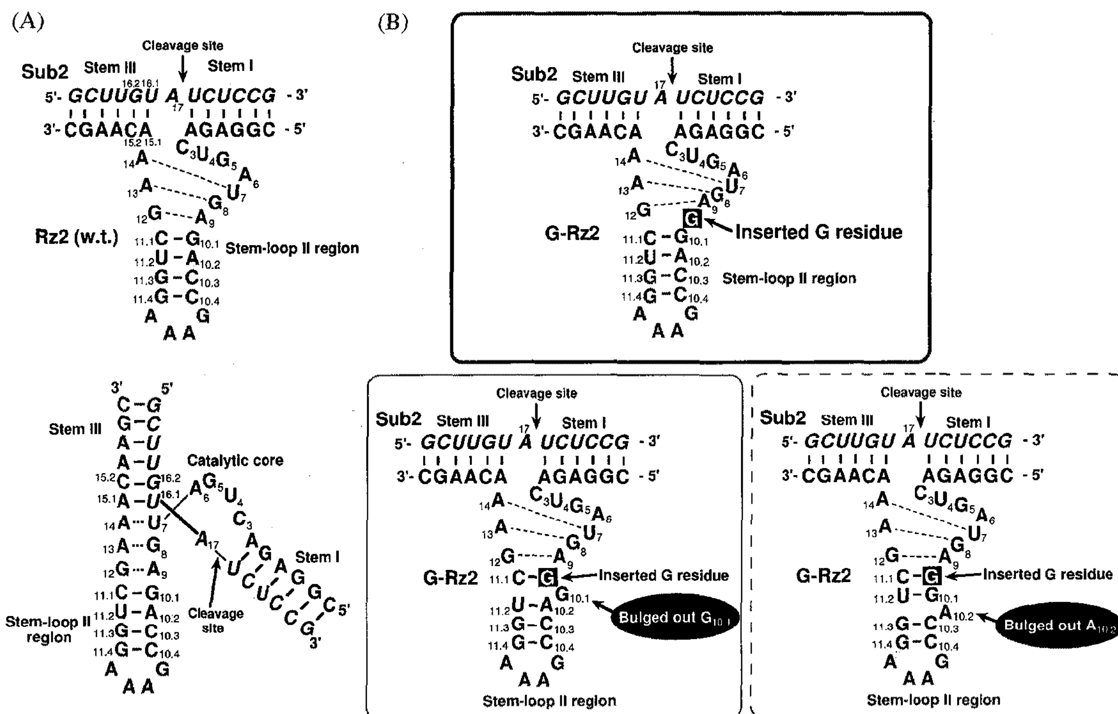


**Fig. 1.** Secondary structures of (A) the wild-type hammerhead ribozyme and (B) the G-inserted mutant hammerhead ribozyme. The substrate-recognition sequence is identical in both Rz2 (w.t.) and G-Rz2.

*et al.*, 1996,1998a; Sugiyama *et al.*, 1996). Such RNA motifs can cleave oligoribonucleotides at specific sites (Koizumi *et al.*, 1988; Ruffer *et al.*, 1990; Perriman *et al.*, 1992; Shimayama *et al.*, 1995; Zoumadakis and Tabler, 1995; Baidya and Uhlenbeck, 1997). In nature, the most commonly found cleavage triplet is GUC, though GUA and AUA have been also observed. Mutagenesis studies have revealed that cleavage triplets of the NUX rule are tolerated (where N is any nucleotide and X is either A, U, or C).

Recently, in order to determine whether other sequences might be possible in the hammerhead's catalytic core, attempts were made to select functional ribozyme sequences *in vitro* from a population of random sequences (Long and Uhlenbeck, 1994; Ishizaka *et al.*, 1995; Thomson *et al.*, 1996; Tang and Breaker, 1997; Vaish *et al.*, 1997; Kore *et al.*, 2000). However, such selection studies indicated that the consensus sequence, derived from viruses and virusoids, was the most optimal sequence (Thomson *et al.*, 1996; Vaish *et al.*, 1997). We reported previously a method for selection of the best target site for ribozyme-mediated cleavage within a fusion gene for adenovirus E1A-associated 300 kDa protein (p300) and luciferase (Kawasaki *et al.*, 1996). Our aim was to construct a simple assay system for the selection of a potential target site of a ribozyme *in vivo* (hereafter, the term ribozymes refers exclusively to hammerhead ribozymes unless otherwise noted). During the construction of the ribozyme vector, we isolated (unexpectedly) an active mutant ribozyme that had an extra G residue inserted between A9 and G10.1 of the consensus catalytic core (Fig. 1). The G-inserted mutant ribozymes had high activities *in vitro* and *in vivo* and they appeared indistinguishable from the wild-type ribozymes (Kawasaki *et al.*, 1996, 1998). This was a surprise to us because attempts in the past failed to isolate any mutant ribozymes that had catalytic activities comparable to that of the wild-type ribozyme (Long and Uhlenbeck, 1994; Ishizaka *et al.*, 1995; Thomson *et al.*, 1996; Tang and Breaker, 1997; Vaish *et al.*, 1997; Kore *et al.*, 2000).

In this report, we examined five types of G-Rz (G-inserted mutant ribozymes), with each G-Rz having a different target site within the p300 mRNA (Fig. 2), and their kinetic parameters were analyzed in terms of the NUX rule (Koizumi *et al.*, 1988; Ruffer *et al.*, 1990; Perriman *et al.*, 1992; Shimayama *et al.*, 1995; Zoumadakis and Tabler, 1995). We found that one type of G-Rz (G-Rz4) had the catalytic power with $k_{cat}$ value of 6.4 $min^{-1}$, in 50 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$. To the best of our knowledge, this is the highest value ever reported for a mutant ribozyme with natural components. These results suggest that the potential activity of a ribozyme might be greater than is usually recognized.

## Materials and methods

**Synthesis of ribozymes and substrates** Mutant ribozymes with G-insertion (G-Rz1~G-Rz5), recently discovered DNA enzymes (Dz2 and Dz5) (Kuwabara *et al.*, 1997; Santoro and Joyce, 1997; Santoro and Joyce, 1998; Warashina *et al.*, 1999) and their corresponding substrates (Sub1~Sub5) were chemically synthesized on a DNA/RNA synthesizer (model 394; Perkin

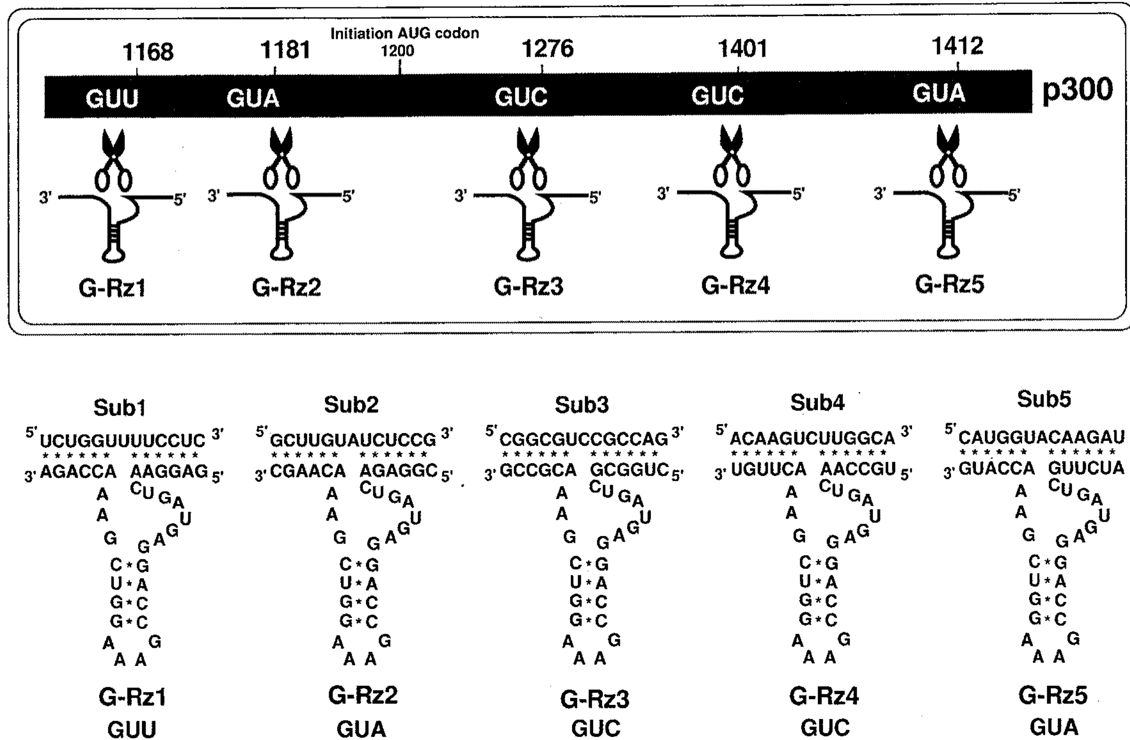


**Fig. 2.** Sequences of G-inserted mutant hammerhead ribozymes and their target sites within the gene for p300. Sequences of their substrates used in kinetic measurements are also shown. The initiation codon (AUG) on p300 mRNA is indicated at nt 1200 and the numbering was referred from Eckner *et al.*, 1994.

Elmer, Applied Biosystems, Foster City, CA). Sub1 contained a GUU triplet located at nt 1168 of the p300 mRNA (Fig. 2) (Eckner *et al.*, 1994). Sub2, 3, 4, 5 contained, respectively, GUA at nt 1181, GUC at 1276, GUC at 1401, and GUA at 1412 (Fig. 2). Reagents for RNA synthesis were purchased from Perkin Elmer, Applied Biosystems. Oligonucleotides were purified as described in the user bulletin from ABI (no. 53; 1989) with minor modifications. Further purification was based on polyacrylamide gel electrophoresis as described previously (Shimayama *et al.*, 1995).

**Kinetic measurements** Reaction rates were measured, in 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0), under substrate-saturating (multiple-turnover) conditions at 37°C. Reactions were usually initiated by the addition of $MgCl_2$ to a buffered solution that contained the enzyme together with the substrate, and each resultant mixture was then incubated at 37°C. The 5' terminus of the substrates were labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (Takara Shuzo, Tokyo, Japan). In all cases, kinetic measurements were made under conditions where all the available ribozyme was expected to form a Michaelis-Menten complex, with high concentrations of substrate (from 100 nM to 10 μM).

Reactions were stopped at intervals by removal of aliquots from the reaction mixture and mixing them with an equal volume of a solution that contained 100 mM EDTA, 9 M urea, 0.1% xylene cyanol and 0.1% bromophenol blue. The substrate and the products of the reaction were separated by electrophoresis on an 8% polyacrylamide/7 M urea denaturing gel and were detected by autoradiography. The extent of cleavage was determined by quantitation of radioactivity in the bands of substrate and products with a Bio-Image Analyzer (BAS2000; Fuji Film, Tokyo).

Cleavage rates were obtained from the slopes of the curves for the time-course of reactions at the initial stage in duplicate at four to five different concentrations of substrate. In the profile of $v$ against $v$/[substrate] (Eadie-Hofstee plots), slopes and intercepts of the plots were determined as $-K_m$ and $k_{cat}$, respectively. A representative graph is shown in Fig. 4C and results of such kinetic analyses are summarized in Table 1.

**Table 1.** Kinetic parameters of wild-type and G-inserted ribozymes and DNA enzymes

| Enzymes | $k_{cat}$ ($min^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ (μM/min) |
|---|---|---|---|
| G-Rz1 | 1.6 | 0.34 | 4.7 |
| G-Rz2 | 1.3 | 0.65 | 2.0 |
| G-Rz3* | 0.020 | 0.18 | 0.11 |
| G-Rz4 | 6.4 | 0.20 | 32 |
| G-Rz5 | 0.75 | 0.19 | 3.9 |
| Dz2* | 0.052 | 7.7 | 0.0068 |
| Dz5* | 0.0013 | 4.0 | 0.00033 |
| Rz2 (w.t.) | 1.0 | 0.1 | 10 |

Rate constants were measured in 50 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ under multiple-turnover conditions at 37°C except for the Rz3*, Dz2* and Dz5*-mediated cleavage reactions which were performed under single-turnover conditions.

## Results and discussion

**Structures of mutant G-inserted ribozymes** Over the past a few years, there have been several attempts to determine both the overall global structure and the detailed atomic structure of the hammerhead ribozyme. The X-ray crystal structures determined by McKay's group and Scott's and Klug's group are nearly identical in terms of tertiary folding and conformation (Pley *et al.*, 1994; Scott *et al.*, 1995, 1996; Murray *et al.*, 1998, 2000). In the X-ray structure (Fig. 1A bottom) the catalytic core is divided into two regions: domain I consisting of $C_3U_4G_5A_6$ and domain II consisting of nucleotides $G_{12}A_{13}A_{14}$ and $U_7G_8A_9$. The nucleotides of domain II form two reversed-Hoogsteen G-A base-pairs between $G_8$-$A_{13}$ and $A_9$-$G_{12}$, and a non-Watson-Crick $A_{14}$-$U_7$ base-pair that consists of one hydrogen bond (these interactions are indicated by dotted lines in Fig. 1). This extended stem II stacks onto the non-Watson-Crick base-pair, $A_{15.1}$-$U_{16.1}$, resulting in formation of a pseudo-A-form helix by stems II and III (Fig. 1A bottom) (Pley *et al.*, 1994; Scott *et al.*, 1995, 1996; McKay, 1996; Scott and Klug; 1996; Murray *et al.*, 1998, 2000). The adjacent non-Watson-Crick A-U base-pairs ($A_{14}$-$U_7$ and $A_{15.1}$-$U_{16.1}$) form the basis of a three-way junction. The four nucleotides ($C_3U_4G_5A_6$) of domain I form a "uridine-turn" motif, allowing the phosphate backbone to turn and connect with stem I.

In the case of our mutant G-inserted ribozymes (G-Rz), the pseudo-A-form helix formed by stems II and III should be perturbed by the insertion of a G residue. We are at preset attempting to probe the secondary structure of G-Rz. Our preliminary data tend to support that the inserted G is bulged out from the pseudo-A-form helix (top structure in Fig. 1B). Even in the absence of the detailed structural information, since G-Rz is expected to show significant cleavage activity, kinetic measurements were made using G-Rz1~G-Rz5 (Fig. 2).

**NUX rule for the G-inserted ribozymes** With respect to the conserved trinucleotide GUC at the cleavage site, results of mutagenic studies revealed that "G" at the third base of the triplet, which might extend stem I by forming a $G_{17}:C_3$ pair, inhibited the cleavage reaction and that a U residue in the central position was required for efficient cleavage (Koizumi *et al.*, 1988; Ruffer *et al.*, 1990; Perriman *et al.*, 1992; Shimayama *et al.*, 1995; Zoumadakis and Tabler, 1995). These observations led to the generally accepted NUX rule (N: A, U, G or C; and X: A, U or C) which states that a substrate with a NUX triplet can be cleaved by a hammerhead ribozyme. In the present study, sequences of the selected five target sites differed not only in the GUX triplet, but also in the substrate-binding site (Fig. 2). In order to examine whether activity of the five G-Rz's might follow the GUX rule, we chemically synthesized five ribozymes (G-Rz1~G-Rz5) and their corresponding substartes (Sub1~Sub5) and kinetic parameters were determined under multiple turnover conditions. Moreover, since we are interested in the target sequence-dependent activity of DNA enzymes (Kuwabara *et al.*, 1997) which were isolated by *in vitro* selection procedure

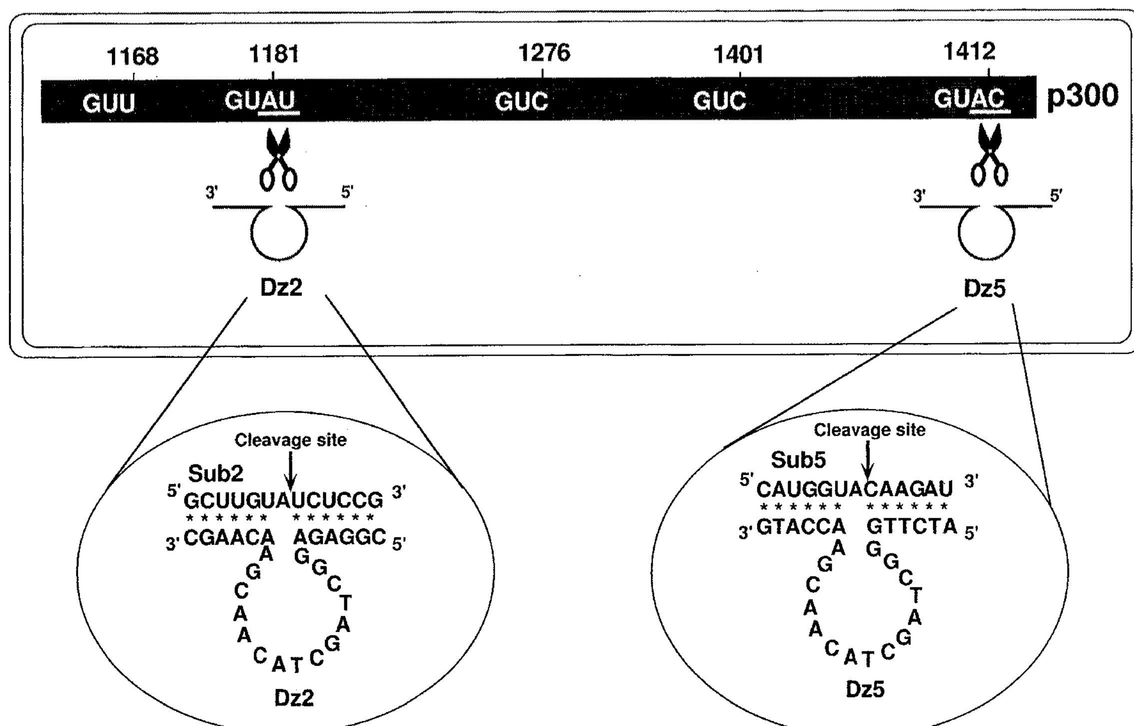


**Fig. 3.** Sequences of DNA enzymes and their substrates. The target sites within the gene for p300 are depicted in the upper panel.

(Santoro and Joyce, 1997), kinetic analysis was also performed on two types of DNA enzyme. These DNA enzymes (Dz2 and Dz5) shared the common cleavage site within Sub2 and the Sub5, respectively, with G-Rz2 and G-Rz5 (Fig. 3). Therefore, direct comparison of kinetic parameters between the DNA enzyme and G-Rz was possible.

Results of such kinetic measurements are summarized in Table 1. Because of the high level of activities of G-Rz, cleavage reactions were performed under multiple turnover conditions. According to our previous analysis of NUX (GUX) rule, with respect to the $k_{cat}$ of substrates with one point mutation at the third base, a "C" residue (wild-type) as the third base is the best, next comes an "A" residue and a "U" residue is the worst, if we ignore the uncleavable substrate with a "G" residue (GU*C*>GU*A*>GU*U*) (Shimayama *et al.*, 1995). With respect to $k_{cat}/K_m$ values in trans reactions, most of the mutant substrates were much less efficiently cleaved than the wild-type substrate with the GU*C* triplet (Shimayama *et al.*, 1995). As can be seen from Table 1, the G-inserted ribozymes basically follow the GUX rule (although not exactly). With respect to both $k_{cat}$ and $k_{cat}/K_m$ values, the GUC triplet (G-Rz4) was much better than the other triplets, such as GUU triplet (G-Rz1) and GUA triplets (G-Rz2 and G-Rz5). In the case of G-Rz3, despite its GUC cleavage site, the activity was the lowest. This is because the substrate (Sub3) sequence is such that it forms intramolecular base-pairings by the bases indicated by italics, 5'-C*GGCG*UC*CGCC*AG-3', to yield a stable hairpin structure. Because of this hairpin structure, kination of Sub3 had to be carried out at an elevated temperature. Because of the low activity of G-Rz3, kinetic parameters of this ribozyme (as well as those of DNA enzymes; see below) were determined under single turnover conditions. The activity of this G-Rz3 based ribozyme was the lowest in HeLa S3 cells as well (Kawasaki *et al.*, 1996).

Concerning the activities of DNA enzymes (Fig. 3), the $k_{cat}$ values were about two orders of magnitude lower than those of the G-inserted ribozymes. Additionally, the $K_m$ values were one order of magnitude higher than those of the ribozymes. Therefore, as a result, the $k_{cat}/K_m$ values were significantly lower than those of the ribozymes. Therefore, as long as these target sites are concerned, ribozymes are more active than the DNA enzymes. It should be mentioned, however, that, in terms of $k_{cat}/K_m$, DNA enzymes were shown previously to be more powerful than hammerhead ribozymes in the cleavage of HIV-1 mRNA (Santoro and Joyce, 1997) and they were also useful *in vivo* (Finkel, 1999; Warashina *et al.*, 1999).

**Activities of the G-inserted ribozymes** As can be seen from Table 1, surprisingly, the $k_{cat}$ value of G-Rz4 was extremely high (6.4 $min^{-1}$). The $k_{cat}$ value of a wild-type ribozyme under similar conditions is in general slightly larger than 1 $min^{-1}$ (Hertel *et al.*, 1997). Our standard ribozyme (R32), which we usually use in kinetic analysis for mechanistic studies because R32 is a well-behaved ribozyme (Zhou and Taira, 1998), has the $k_{cat}$ value of 2.5 $min^{-1}$ under

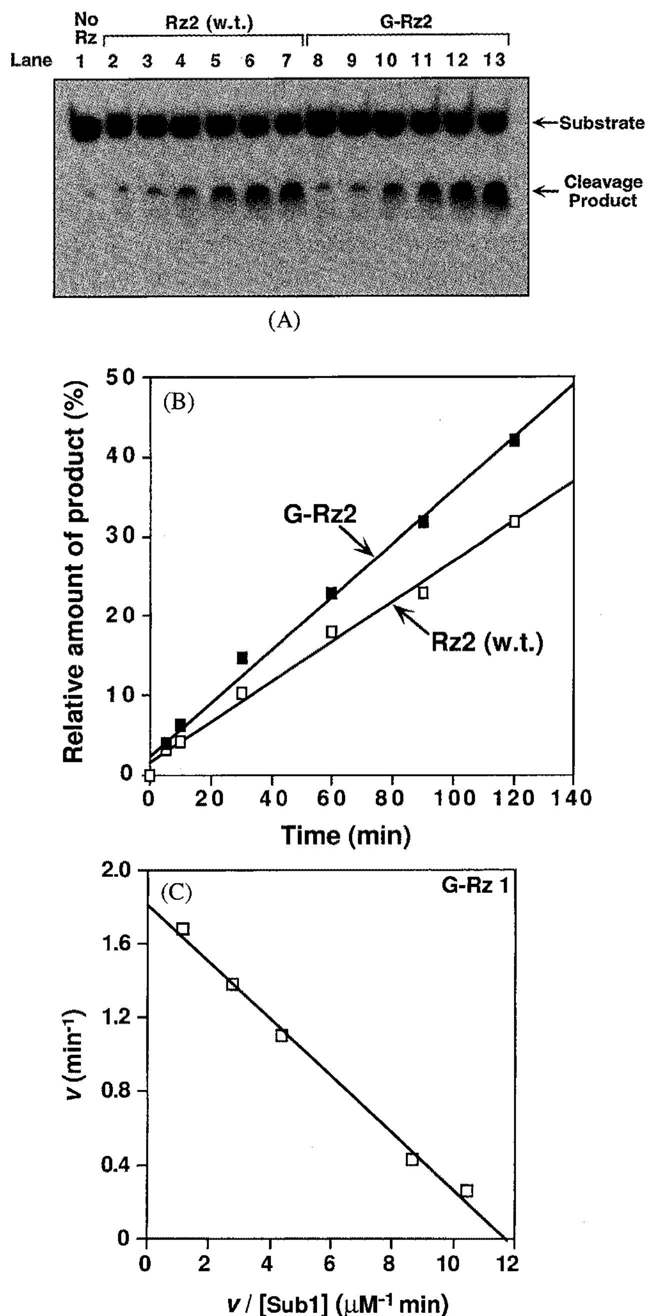


**Fig. 4.** Comparison of activities of the hammerhead ribozyme [Rz2 (w.t.)] and the G-inserted hammerhead ribozyme (G-Rz2). (A) Cleavage activity of the Rz2 (w.t.) and the G-Rz2. Bands in each lane represent the reaction product formed in 5 min (lane 2, 8), 10 min (lane 3, 9), 30 min (lane 4, 10), 60 min (lane 5, 11), 90 min (lane 6, 12), and 120 min (lane 7, 13) at 37°C in 50 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$. Concentrations: Sub2 with a trace amount of 5'-$^{32}$P-labeling, 0.5 μM; Rz2 (w.t.), 5 nM; G-Rz2, 5 nM. Lane 1, control (no ribozyme); lanes 2 to 7, cleavage products by Rz2 (w.t.); lane 8 to 13, cleavage products by G-Rz2. (B) Time courses of cleavage reactions by Rz2 (w.t.) and G-Rz2. Open squares; Rz2 (w.t.). Closed squares; G-Rz2. (C) Eadie-Hofstee plot of a set of data obtained under multiple-turnover conditions for G-Rz 1. Calculated values of $k_{cat}$ and $K_m$ are summarized in Table 1.

the conditions used in this study. To the best of our knowledge, G-Rz4 appears to be the most active mutant ribozyme that has the natural all-RNA backbones. However, our previous study demonstrated that the repression activity of the G-Rz4 based ribozyme against p300 gene *in vivo* was lower than that of the G-Rz2 based ribozyme (Kawasaki *et al.*, 1996). This is because the rate-limiting step for the cleavage of p300 mRNA *in vivo* is unlikely to be the chemical step. The G-Rz2 based ribozyme was extremely active *in vivo* and, therefore, this ribozyme technology could successfully lead to the conclusion that p300 and CBP (CREB binding protein) play a separate function during the RA (retinoic acid)-induced differentiation and apoptosis (Kawasaki *et al.*, 1998).

We compared the activity *in vitro* of G-Rz2 and its parental wild-type ribozyme [Rz2 (w.t.) in Fig. 1A] and the results are shown in Figure 4 and Table 1. In terms of $k_{cat}$, G-inserted ribozyme was slightly more active than the wild-type ribozyme (Fig. 4). However, in terms of $k_{cat}/K_m$, the wild-type ribozyme was 5-fold more active than the G-inserted ribozyme (Table 1). Present study clearly demonstrates that some G-Rz · substrate complexes are in a more favorable position to reach the transition state than the wild-type ribozyme · substrate complex. This finding seems to suggest that it might be possible to select *in vitro* or *in vivo*, or to engineer, hammerhead ribozymes (Beaudry and Joyce, 1992; Pan and Uhlenbeck, 1992; Lehman and Joyce, 1993; Long and Uhlenbeck, 1994; Nakamaye and Eckstein, 1994; Cuenoud and Szostak, 1995; Ishizaka *et al.*, 1995; Usman *et al.*, 1996; Breaker, 1997; Fujita *et al.*, 1997; Tang and Breaker, 1997; Vaish *et al.*, 1997; Kore *et al.*, 2000) that are better catalysts than wild-type forms (Kurata *et al.*, 2000; Kuwabara *et al.*, 1998a,b, 1999, 2000; Tanabe *et al.*, 2000).

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