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Generality of the NUX Rule: Kinetic Analysis of the Results of Systematic Mutations in the Trinucleotide at the Cleavage Site of Hammerhead Ribozymes

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ABSTRACT: In order to study in detail the generality of the NUX (N = A, U, G, or C; X = A, U, or C) rule for the GUC triplet adjacent to the cleavage site in hammerhead ribozymes, two kinetic parameters, namely, k_{cat} and K_m, were determined for substrates with mutations in this triplet, which included double mutants with mutations of both N and X. All substrates with mutated cleavage sites were cleaved with reduced efficiency compared to the wild type. However, some mutations mainly affected k_{cat} and others mainly affected K_m, a phenomenon that could not have been predicted from previous results. *A* as the first or third base increased K_m by 35- or 30-fold, respectively, while the effect on k_{cat} was small. *U* as the first or third base decreased k_{cat} by 8- or 15-fold, respectively, while the effect on K_m was small. The effect of *C* as the first base on kinetic parameters was relatively small. The kinetic parameters of double mutants generally were determined by the effects of both individual point mutations. The *AUA* triplet gave a very much higher k_{cat} than the other double mutants tested. In general, all of the mutants except for the mutant substrate with the *CUC* triplet had very low cleavage efficiencies, which ranged from 0.6% to 8% of the wild-type value, as a result of the deleterious effects of the mutations on k_{cat}, K_m, or both. This kind of analysis can explain why nature has chosen triplets such as GUC, GUA, and AUA in *cis* reactions: all of these triplets retain high k_{cat} values, although the K_m values of GUA and AUA are high.

Hammerhead ribozymes are RNA molecules consisting of a catalytic loop and three stems (I, II, and III) that can cut RNAs with high specificity (they normally cleave on the 3′-side of the GUC triplet; Figure 1). They act "in *cis*" in nature, and well-characterized examples include the hammerheads in (+)sTRSV[1] (Buzayan et al., 1986; Prody et al., 1986), ASBV (Huchins et al., 1986), vLTSV (Forster & Symons, 1987), and the newt (Epstein & Gall, 1987). However, they have also been engineered such that they can act "in *trans*". This property has been achieved in two ways. In one case, the hammerhead domain was divided between stems I and II (Uhlenbeck, 1987), and in another it was divided between stems I and III (Haseloff & Gerlach, 1988; these two in *trans* reaction systems are, hereafter, designated *trans* 1 and *trans* 2, respectively). In order to define the sequence requirements for the hammerhead structure, extensive mutagenesis studies of the conserved region have been performed in *cis* (Sheldon & Symons, 1989), *trans* 1 (Koizumi & Ohtsuka 1988; Ruffner et al., 1990), and *trans* 2 (Perriman et al., 1992) reaction systems.

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 (**2**, **3**, and **4** in Table 1A), except in one case (**1** in Table 1A), and that a U residue in the central position was required for efficient cleavage (Koizumi & Ohtsuka, 1988; Ruffner et al., 1990; Perriman et al., 1992). These observations led to the generally accepted NUX rule (N = A, U, G, or C; X = A, U, or C), which states that a substrate with an NUX triplet can be cleaved by a hammerhead ribozyme. However, there are some inconsistencies among the results reported by different groups (Table 1A). For example, a substrate that contained the *AUC* triplet was cleaved in one study with efficiency comparable to cleavage of the wild type (Table 1A **3**; Ruffner et al., 1990), while in other studies, that substrate was cleaved with much lower efficiency (Table 1A, **2**; Koizumi & Ohtsuka, 1988) or no cleavage was observed (Table 1A, **4**; Perriman et al., 1992). The differences may be due not only to differences among reaction systems, which include the type of reaction (*cis* or *trans* and also *trans* 1 or *trans* 2), the sequence of the hammerhead complex, and the reaction conditions, but also to the experimental design: in every case, only a simple comparison of the cleavage activities of certain substrates at fixed concentrations was performed. Even though previous results were determined with ribozyme at a 1.3−1.5-fold molar excess compared to the substrate, the observed rate of cleavage would be lower than the maximum rate (k_{cat}) unless the total concentration of substrate and ribozyme were high enough with respect to the K_m value. In other words, the rate constants determined could reflect either k_{cat} or k_{cat}/K_m values, depending on the

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[1] Abbreviations: ASBV, avocado sunblotch viroid; (+)sBYDV, plus strand of the satellite RNA of barley yellow dwarf virus; (+)- or (−)-vLTSV, plus or minus strand of the virusoid of lucerne transient streak virus; (+)sTRSV, plus strand of the satellite RNA of tobacco ringspot virus. Mutated bases and bases in the triplet to which the reader's attention is drawn are indicated by italics, except in Table 1 where mutated bases are indicated by larger letters.

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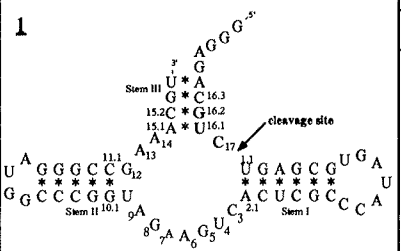
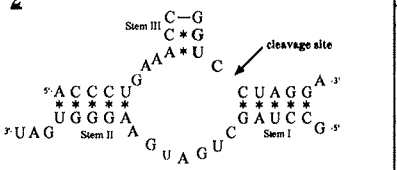
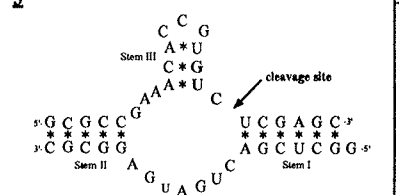
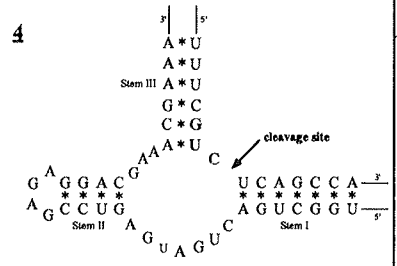
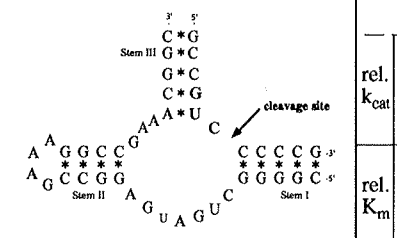
Table 1: Results of Point Mutagenesis of the GUC Triplet at the Cleavage Site of Hammerhead Ribozymes: (A) Mutagenesis Analysis Reported in the Past; (B) Mutagenesis Analysis of Our Own

	Hammerhead structure[a]	Relative activities of the point mutants[b]					
		AUC	UUC	CUC	GUA	GUU	GUG
A		During transcription[c]					
1		NM[d]	NM	NM	NM	1	0.3
		23 h cleavage[e]					
2		0.1	0.4	1	1	1	0
		Rate constants[f]					
3		0.57	0.25	0.083	0.7	0.05	<0.003
		1 h cleavage[g]					
4		0	1.2	0.95	0.93	0.68	0
B		kcat and Km[h]					
		rel. k_{cat}	1.1	0.13	0.48	0.60	0.065	0
		rel. K_m	35	1.7	2.5	30	1.7	

[a] The sequences of hammerheads used are shown. The GUC triplet is indicated in boldface letters. The unconserved base numbered 7 in the catalytic loop of each ribozyme is written in small print [the numbering of the bases is in accord with the nomenclature of Hertel et al. (1992)]. The asterisks indicate base pairings. [b] The values presented are observed activities relative to that of each individual wild-type substrate with the GUC triplet: the latter value is taken as 1. Mutated bases in the triplet are indicated in larger print. [c] *Cis* reaction (Sheldon & Symons, 1989) adopted from (+)vLTSV (Forster & Symons, 1987). Its structural features are as follows: (i) the catalytic loop has an additional U between A_9 and $G_{10.1}$, and (ii) the catalytic loop has A_7 as the unconserved base (normally U_7). The cleavage reaction occurred simultaneously with transcription by T7 RNA polymerase. [d] NM means that the cleavage activity of the corresponding mutant was not determined. [e] *Trans* 1 reaction (Koizumi & Ohtsuka, 1988) from newt (Epstein & Gall, 1987). There is an $A_{10.1}$:$U_{11.1}$ base pair in stem II (normally a G:C base pair), which is known to be sensitive to mutation (Ruffner et al., 1990; Tuschl & Eckstein, 1993). The extent of cleavage was analyzed after a 23 h incubation in a solution that contained 1.1 μM substrate, 1.6 μM ribozyme, 25 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5), and 20 mM NaCl at 37 °C. [f] *Trans* 1 reaction (Ruffner et al., 1990) conditions were as follows: 0.2 μM substrate, 0.3 μM ribozyme, 10 mM $MgCl_2$, and 50 mM Tris-HCl (pH 8) at 37 °C. Samples were analyzed at five time points, giving 0−60% total cleavage, and the cleavage half-lives ($t_{1/2}$) were used to obtain first-order rate constants. [g] *Trans* 2 reaction (Perriman et al., 1992) in which the substrate used was 80 nucleotides long. The extent of cleavage was analyzed after a 1 h incubation in a solution that contained 0.03 μM substrate, 0.04 μM ribozyme, 10 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.4) at 50 °C. [h] Our *trans* 2 reaction conditions were as follows: 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0) at 37 °C. The values presented are values of k_{cat} and K_m relative to the values for the wild-type substrate with the GUC triplet.

concentrations of substrate and ribozyme used in the experiments. Therefore, it is important to determine whether a reduction in rate is caused by a reduced k_{cat} value or by an increase in the K_m value. Furthermore, detailed kinetic analysis on k_{cat} and K_m values of the mutants can distinguish, to some extent, whether the change in activity originates from the intrinsic property of the mutants or from other factors, such as inactive complex formation. Thus, the determination of individual k_{cat} and K_m values is essential for an objective discussion of differences in activity between various mutant forms of the cleavage site.

From a practical point of view, since *trans* 2 reactions are suitable for targeting certain RNAs (Haseloff & Gerlach, 1988; Sarver et al., 1990), a detailed examination of the NUX rule in *trans* 2 systems should help us to choose target sequences other than GUC. However, in the case of *trans* 2 system, only one study of point mutagenesis of the NUX triplet has been reported (Perriman et al., 1992). Furthermore, no systematic analysis of double mutants at the first and third positions has been reported, even though a few double mutants at the first and second positions (Perriman et al., 1992) or at the first and third positions (Nakamaye & Eckstein, 1994) have been created and reported to show slow but discernible cleavage. Therefore, in order to investigate the range of cleavage activity of the NUX triplets, we performed detailed kinetic analysis of all possible NUX mutants by measuring individual k_{cat} and K_m values.

MATERIALS AND METHODS

Materials. Substrates and ribozymes for cleavage reactions catalyzed by hammerheads were synthesized chemically on a DNA synthesizer (Model 392, Applied Biosystems, Inc. (ABI), Foster City, CA). RNA-related reagents were purchased from American Bionetics, Inc. (ABN, Hayward, CA). Other reagents were purchased from either ABI or ABN. Purification of synthesized oligonucleotides has been described elsewhere (Shimayama et al., 1993a).

Kinetic Measurements. The conditions for the cleavage reaction were 25 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0), and 37 °C. Concentrations of RNAs were determined by measuring absorbance at 260 nm. Solutions of ^{32}P-5′-end-labeled substrate and non-radiolabeled ribozyme [final concentrations, 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0)] were preincubated separately at 37 °C. Each reaction was initiated by the addition of substrate to a solution of ribozyme in a final volume of 10 μL. Final concentrations of substrates ranged from 0.02 to 3.5 μM and those of ribozymes ranged from 1.4 to 220 nM. Conditions were adjusted such that 20−60% of each substrate was cleaved in about 15 min, and samples were removed for analysis at four time points. These samples were quenched by mixing them with an equivalent volume of a solution of 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue, with subsequent snap-cooling on ice. Intact substrate and products of cleavage were separated by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel and were detected by autoradiography. The extent of cleavage was determined from measurements of radioactivity in the bands of the intact substrate and the 5′-product, obtained with a Bio-Image Analyzer (BA100 or BAS2000, Fuji Photo Film Co. Ltd., Tokyo, Japan). The simple Michaelis−Menten equation was used for kinetic analysis.

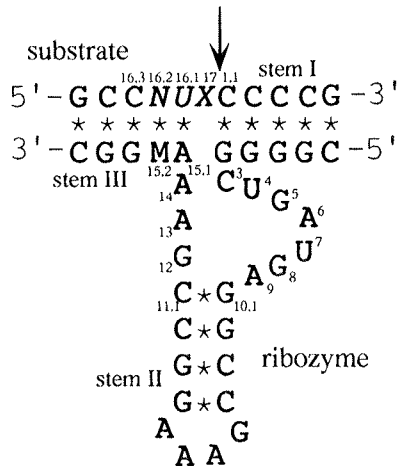


FIGURE 1: Structure of hammerhead ribozymes used in this study of the *trans* 2 cleavage reaction. The numbering of the bases is in accord with the nomenclature of Hertel et al. (1992). The NUX triplet in the substrate is shown in italics: N is any nucleotide, X is any nucleotide except G, and M in the ribozyme is any nucleotide that is complementary to N. Asterisks indicate base pairings, and an arrow indicates the cleavage site.

Values of K_m and V_{max} were derived from both Eadie−Hofstee and Lineweaver−Burk plots. The k_{cat} values were calculated as V_{max} divided by the concentration of ribozyme.

RESULTS

Cleavage Reaction System of the Hammerhead Ribozyme. The hammerhead structure that we used is based on a *trans* 2 system (Figure 1). The wild-type values of k_{cat} and K_m in our system were previously determined to be 4.0 min^{-1} and 20 nM, respectively (Shimayama et al., 1993a). The k_{cat} value was proved previously to represent the rate of chemical cleavage and not the rate of product release, by the absence of burst kinetics (Sawata et al., 1993).

In this study, we prepared 11 different mutated substrates by reference to the NUX rule: we maintained a U residue as the second base, and no G residue was included as the third base because these features represent the minimum requirement for the postulated NUX rule associated with ribozyme activity. In fact, the mutant with a GU*G* triplet was also uncleavable in our case (the actual k_{cat} value for the GU*G* triplet measured under the ribozyme-saturating single-turnover conditions was <0.002 min^{-1}, which we indicate as no cleavage in Table 1B). Three kinds of mutated ribozyme were similarly prepared so that all of the substrates to be examined maintained the $N_{16.2}$:$M_{15.2}$ base pair (Figure 1).

Kinetic Analysis after Point Mutations in the GUC Triplet. The results of point mutagenesis are presented in Table 1B and in the upper part of Table 2. An *A* residue as the first or third base increased the K_m value by 35- and 30-fold, respectively, while the effect on k_{cat} was small. These results suggest that substrates with *A*UC and GU*A* triplets, because of their higher K_m values, would be very poorly cleaved under low substrate (k_{cat}/K_m) conditions, while they would be cleaved with efficiency comparable to that of the wild type (GUC triplet) under substrate-saturating (k_{cat}) conditions. In contrast to an *A* residue, a *U* residue as the first or third base decreased k_{cat} by 8- and 15-fold, respectively, while the effect on K_m was small. In fact *U*UC and GU*U* triplets

Table 2: Kinetic Parameters of Mutant Substrates with Changes in the NUX Triplet[a]

triplet[b]	k_{cat}, (min^{-1})	K_m, (nM)	k_{cat}/K_m (μM^{-1} min^{-1})	relative k_{cat}/K_m
GUC[c]	4.0	20	200	1
*A*UC	4.4	700	6.3	0.032
*U*UC	0.52	33	16	0.080
*C*UC	1.9	50	38	0.19
GU*A*	2.4	600	4.0	0.020
GU*U*	0.26	34	7.6	0.038
*A*U*A*	2.1	320	6.6	0.033
*A*U*U*	0.53	320	1.7	0.0085
*C*U*A*	0.16	64	2.5	0.013
*C*U*U*	0.050	45	1.1	0.0055
*U*U*A*	0.39	140	2.8	0.014
*U*U*U*	0.10	33	3.3	0.015

[a] The hammerhead structure used for this study is shown in Figure 1, and the conditions of kinetic analysis are described in Materials and Methods. [b] Mutated nucleotides are indicated in italics. [c] The GUC triplet is the wild-type sequence and its kinetic parameters were determined previously (Shimayama et al., 1993a). The upper part of the table shows the results of point mutations and the lower part shows those of double mutations.

Table 3: Comparison of the Kinetic Parameters of Substrates with Double Mutants and the Corresponding Single Mutants in the NUX Triplet[a]

k_{cat} (min^{-1})			K_m (nM)		
double mutation	point mutation		double mutation	point mutation	
*A*U*A*	*A*UC	GU*A*	*A*U*A*	*A*UC	GU*A*
2.1	4.4	2.4	320	700	600
*A*U*U*	*A*UC	GU*U*	*A*U*U*	*A*UC	GU*U*
0.53	4.4	0.26	320	700	34
*C*U*A*	*C*UC	GU*A*	*C*U*A*	*C*UC	GU*A*
0.16	1.9	2.4	64	50	600
*C*U*U*	*C*UC	GU*U*	*C*U*U*	*C*UC	GU*U*
0.050	1.9	0.26	45	50	34
*U*U*A*	*U*UC	GU*A*	*U*U*A*	*U*UC	GU*A*
0.39	0.52	2.4	140	33	600
*U*U*U*	*U*UC	GU*U*	*U*U*U*	*U*UC	GU*U*
0.10	0.52	0.26	33	33	34

[a] The hammerhead structure used for this study is shown in Figure 1, and the conditions of kinetic analysis are described in Materials and Methods. Mutated bases are indicated in italics. Values of k_{cat} and K_m for the wild-type GUC triplet were 4.0 min^{-1} and 20 nM, respectively (Shimayama et al., 1993a).

gave the lowest and the second lowest k_{cat} values among substrates with one point mutation. The effect of a *C* residue as the first base on the efficiency of cleavage was relatively small because the changes in both k_{cat} and K_m were relatively small. Indeed, the substrate with the *C*UC triplet gave the best value as measured by k_{cat}/K_m among all of the NUX mutants.

The substrate with an *A*UC triplet gave a slightly higher k_{cat} than that of the wild type (GUC); a substrate that contained either *G*UC or *A*UC was cleaved at almost the same rate under k_{cat}-controlled conditions, despite the unfavorable K_m value of the *A*UC substrate. Therefore, with respect to the k_{cat} after one point mutation at the first base, *G* and *A* residues are the best, next comes a *C* residue, and a *U* residue yields the worst substrate (*G*UC, *A*UC > *C*UC > *U*UC). 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*). With respect to k_{cat}/K_m, all of the mutants except for the mutant with the *C*UC triplet were poorly cleaved (from 2% to 8% of the wild-type activity) as a result of disadvantageous effects on k_{cat} or K_m (Table 2).

Kinetic Analysis of Substrates with a Double Mutation at the First and Third Bases of the GUC Triplet. Results of double mutagenesis are presented in the lower part of Table 2. Table 3 shows the comparison of k_{cat} and K_m values for double mutants and those of corresponding point mutants. From the comparison, the k_{cat} values of double mutants are generally smaller than those of corresponding individual point mutants, while the K_m values of double mutants are generally intermediate between those of corresponding individual point mutants. In general, all of the double mutants were very poor substrates, with cleavage efficiencies from 0.6% to 3% of the wild-type activity, as a result of the disadvantageous effects of mutations on k_{cat}, for the most part, or on both k_{cat} and K_m.

With respect to k_{cat}, all of the double mutants, except for a few cases such as the substrate with an *A*U*A* triplet, for example, gave very low values, as they are affected by the decreases due to both individual point mutations (Table 3). The substrate with the *A*U*A* triplet gave an exceptionally high k_{cat} among the double mutants, and this result agrees well with the results of point mutants *A*UC and GU*A* with relatively high k_{cat} values (Tables 2 and 3). In terms of the k_{cat} values of double mutations, an *A* residue as the first base gives the best substrate, next comes a *U* residue, and a *C* residue is the worst (*A*U*A* > *U*U*A* > *C*U*A*; *A*U*U* > *U*U*U* > *C*U*U*); this trend is slightly different from that observed for a single point mutation at the first base (*G*, *A* > *C* > *U*). As found in the case of one point mutation, *A* as the third base is always better than *U* for a favorable k_{cat} (*A*U*A* > *A*U*U*; *C*U*A* > *C*U*U*; *U*U*A* > *U*U*U*). In general, with respect to the third position of the triplet, a *U* residue is always the worst base in terms of k_{cat}. It may be that U_{17} and A_{14} can partially base pair, as do G_{17} and C_3, perhaps with resultant inhibition of cleavage.

Since the k_{cat} values of double mutations were more or less synergistically affected by the corresponding single mutations, while the K_m values of double mutants generally showed the intermediate value of the corresponding single mutations, the reductions in cleavage efficiency of double-mutant substrates were primarily caused by the result of lower k_{cat} values.

DISCUSSION

Analysis of the Validity of the NUX Rule in Terms of k_{cat} and K_m. The generally accepted NUX rule is, in some cases, misleading. In selecting a target sequence for hammerhead ribozyme-catalyzed cleavage, one might assume that any sequence that conforms to the NUX rule would be useful. In fact, in the past, we chose a relatively conserved region of HIV-1 as the cleavage site that contained a *C*U*U* triplet (Shimayama et al., 1993b). We assumed, on the basis of results of mutagenesis using *C*UC (95% activity) and GU*U* (68% activity) in the *trans* 2 system (Table 1A), that the cleavage after *C*U*U* would occur at a reasonable rate. However, the cleavage reaction was very slow. Since the NUX rule was derived from the results of point mutations, and since the effect of various mutations on k_{cat} and K_m was

unknown, we felt it necessary to examine systematically the effects of single and double mutations in the relevant triplet of the substrate on k_{cat} and K_m.

We previously constructed a hammerhead ribozyme that functions in a *trans* 2 reaction system (Taira & Nishikawa, 1992), as shown in Table 1B and Figure 1. Its structure was based on (−)vLTSV (Forster & Symons, 1987). In order to avoid kinetic complexities, the substrate sequence was carefully designed so that unfavorable intra- and intermolecular base pairing of substrate and ribozyme would be avoided. Indeed, our substrates were cleaved almost completely without any indication of the formation of an inactive complex (Shimayama et al., 1993a; Sawata et al., 1993). We selected a substrate 11 nucleotides in length (Taira & Nishikawa, 1992) because, were a longer binding sequence to be used, the product release step rather than the chemical cleavage step might become the rate-limiting step (Fedor & Uhlenbeck, 1992). Our simplified and kinetically defined system facilitates the interpretation of kinetic parameters of reactions with mutant substrates.

We analyzed for the first time the effects of mutations, including double mutations, in terms of the k_{cat} and K_m values of each mutant. In this kind of quantitative analysis, we need to know what we are measuring as the observed rate constant, k_{obs}. In general, depending on the conditions of the measurements, k_{obs} can potentially reflect (i) unfolding of the substrate or the ribozyme prior to the formation of a complex, (ii) association of the ribozyme and substrate, (iii) a conformational change in the ribozyme−substrate complex, (iv) the chemical cleavage step, (v) the product dissociation step, etc. Since we were interested in the chemical cleavage step with each mutant substrate in this study, we chose a short substrate sequence that would avoid any formation of an inactive complex, and we chose the experimental conditions carefully to avoid the possibility of determining the rate of the reaction by processes i, ii, iii, and v.

The variations in k_{cat} and K_m values among mutants in our system generally were not due to the formation of an inactive complex. We checked whether such an inactive complex might form upon the introduction of mutations. Among the various mutants we created, with respect to "false" base pairing between the substrate and the catalytic loop of the ribozyme, four base pairs were maximally possible only for the mutated substrate with the *A*UC triplet (5′-$C_{16.3}A_{16.2}U_{16.1}C_{17}$-3′/5′-$G_5A_6U_7G_8$-3′; see Figure 1). However, this kind of interaction was unlikely because, if it were to occur, since the concentration of the active ribozyme−substrate complex would be reduced, alterations in the values of both k_{cat} and K_m would be observed simultaneously that could not be fitted to our results for the *A*UC triplet (Table 2: only K_m was altered). Regarding inactivation via intersubstrate aggregation, a maximum of four base pairs was possible for the mutated substrate that contained the GU*A* triplet (5′-$G_{16.2}U_{16.1}A_{17}C_{1.1}$-3′; see Figure 1). However, even in this case, no substrate inhibition was observed, at least at the highest concentration checked in the kinetic analysis of the GU*A* mutant (the linearity in the Eadie−Hofstee plot was maintained over the entire range of substrate concentrations used; data not shown).

While our studies on the mutant triplets were based on a single substrate sequence, we consider that the effects of the base compositions of helix I and helix III are relatively small because (i) we previously observed a similar, very low k_{cat} value of about 0.06 min^{-1} in model reactions where we targeted a *CUU* triplet with different substrate sequences (we targeted at the LTR region of HIV-1; Shimayama et al., 1993b), and this value is nearly identical to the value (0.05 min^{-1}) obtained in this study and (ii) almost identical values of k_{cat} were obtained previously with several hammerheads that contained identical catalytic core sequences but differed in the base compositions of helix I and helix III, with differences of about 60-fold in values of K_m due to the formation of intra- or intersubstrate complexes that were nonreactive (Fedor & Uhlenbeck, 1990). Therefore, at present, our analysis provides the most useful information regarding the NUX rule.

Comparison with Other Systems. According to our results, the NUX rule seems general when it is considered in the light of cleavable or not cleavable substrates, because all of the mutants, including the double mutants, were cleaved to some extent, but the efficiency, as measured by values of k_{cat}/K_m, was very low except in the case of the *C*UC triplet (Tables 1B and 2). According to our results with point mutations, the mutants could be divided into three groups: (i) a group in which mainly the K_m value was changed (*A*UC and GU*A*); (ii) a group in which mainly the k_{cat} value was changed (*U*UC and GU*U*); and (iii) a group in which the changes in both values were relatively small (*C*UC).

A striking difference in the effects of point mutations among the systems examined by different research groups is the fact that the *A*UC triplet, which gave a relatively high k_{cat} value in our system, was cleaved with very low efficiency (Table 1A, **2**; Koizumi & Ohtsuka, 1988) or not at all (Table 1A, **4**; Perriman et al., 1992) in studies by others. This discrepancy may be due, at least in part, to the increase in K_m, according to our results. The hammerhead complex had a shorter stem III in the former case (Table 1A, **2**), and the cleavage reaction was performed at 50 °C in the latter case (Table 1A, **4**). In both cases, the reaction with a higher K_m would be very disadvantageous. Furthermore, the observation of no cleavage activity in the latter case and our observation of a much higher K_m for the *A*UC triplet suggest that, at the higher temperature of 50 °C, the $A_{16.2}$:$U_{15.2}$ and $U_{16.1}$:$A_{15.1}$ base pairs might have melted to produce an inactive structure, even though the rest of the base pairs in the long stem I and stem III could have been maintained.

The general discrepancy between our results and those of Perriman et al. (1992; Table 1A, **4**), with measurements in both cases being based on the same *trans* 2 reaction, may be due to the difference in the length of the substrate: their substrate was much longer (80 bases) than ours, and self-aggregation of both substrate and ribozyme could be anticipated from predictions of RNA secondary structure (Zuker, 1989; Jaeger et al., 1989; data not shown), as originally stated by the investigators. The unfolding and association between a correctly aligned ribozyme and substrate potentially could become the rate-determining step, at least in part. Therefore, as we stated earlier, a longer substrate might complicate analysis of the kinetics. Indeed, in the case of cleavage reactions with longer substrates such as mRNAs, the observed rates of reaction were much lower than those with the corresponding short substrates (Heidenreich & Eckstein, 1992). This was also true for a ribozyme with an extra sequence (Chowrira et al., 1994). When a longer binding sequence is used for kinetic analysis, disadvantageous low cleavage activities of the mutants could be

masked by processes other than the chemical cleavage step.

In the case of Ruffener et al. (1990; Table 1A, **3**), the rate constants presented seemed to almost represent the k_{cat} values because the total concentrations of the substrate and the ribozyme were high (0.5 μM; see the footnotes for Table 1) and also because the rate constants were obtained under single-turnover conditions. In fact, their relative reaction rates of one-point mutants (Table 1A, **3**) are in accord with our relative cleavage rates (Table 1B), except for the *C*UC triplet. The *C*UC triplet, in their case, gave exceptionally low cleavage activity. Their sequence with the *C*UC triplet is capable of forming an inactive structure (stem III with its loop of five bases) that, we believe, is the cause of the low activity of their *C*UC mutant.

Recently, results of the cleavage of substrates with AUN triplets in *cis* and also in the *trans* 2 reaction system were reported that are not necessarily in accord with our results (Nakamaye & Eckstein, 1994). Analysis of the reported sequence reveals that an alternative inactive structure, in which part of the catalytic loop base pairs with the substrate, can be predicted for the reported sequence (Zuker, 1989; Jaeger et al., 1989; data not shown). This structure might explain the discrepancy between the reported results and ours.

Importance of the NUX Triplet. The reason for the increase in K_m with an *A* residue at the first position does not seem to be merely a weaker $A_{16.2}$:$U_{15.2}$ base pair since *U* as the first base barely affected the K_m (Table 2). The fact that not only the third base of the triplet that was adjacent to the cleavage site but also the first base, which was relatively far from the cleavage site, affected both k_{cat} and K_m suggests that the contribution of the triplet to the establishment of an active structure might be important. In support of this hypothesis, we found previously, in order to maintain the full activity in this system, that it is important to keep the first base of G as a ribonucleotide, suggesting that the first position (N) of the NUX triplet is also important for cleavage activity (Shimayama et al., 1992; Shimayama, 1994). It should also be noted that the effects of the mutation at the first base ($N_{16.2}$) of the triplet might include those of the corresponding counterbase ($M_{15.2}$), which must be automatically mutated for base pairing with the first base (Figure 1).

Although, 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 GUC triplet, the *A*UC, *C*UC, GU*A*, and even double-mutated *AUA* triplets gave k_{cat} values similar to that of the substrate with the GUC triplet. Therefore, these mutants should be well cleaved under k_{cat}-controlled conditions, where the concentrations of substrate and/or ribozyme are sufficiently high compared to K_m. In fact, (−)vLTSV and (+)sBYDV use the GU*A* and the *AUA* triplets, respectively (Forster & Symons, 1987; Miller & Silver, 1991), for hammerhead cleavage during their replicating process. In these *cis* reactions, K_m values are irrelevant.

Our results, as summarized in Table 2, should be useful in choosing the target sequences for hammerhead cleavage reactions and also in studies of the cleavage mechanism.

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