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Specificity of hammerhead ribozyme cleavage

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To be effective in gene inactivation, the hammerhead ribozyme must cleave a complementary RNA target without deleterious effects from cleaving non-target RNAs that contain mismatches and shorter stretches of complementarity. The specificity of hammerhead cleavage was evaluated using HH16, a well-characterized ribozyme designed to cleave a target of 17 residues. Under standard reaction conditions, HH16 is unable to discriminate between its full-length substrate and 3′-truncated substrates, even when six fewer base pairs are formed between HH16 and the substrate. This striking lack of specificity arises because all the substrates bind to the ribozyme with sufficient affinity so that cleavage occurs before their affinity differences are manifested. In contrast, HH16 does exhibit high specificity towards certain 3′-truncated versions of altered substrates that either also contain a single base mismatch or are shortened at the 5′ end. In addition, the specificity of HH16 is improved in the presence of p7 nucleocapsid protein from human immunodeficiency virus (HIV)-1, which accelerates the association and dissociation of RNA helices. These results support the view that the hammerhead has an intrinsic ability to discriminate against incorrect bases, but emphasizes that the high specificity is only observed in a certain range of helix lengths.

Keywords: antisense/enzyme kinetics/HIV nucleocapsid/ RNA catalysis

Introduction

The hammerhead is a small RNA motif consisting of three helices and an 11 nucleotide core that causes rapid cleavage of the RNA chain at a unique site (Buzayan *et al.*, 1986; Forster and Symons, 1987a). By assembling the hammerhead from two RNA molecules through the formation of intermolecular base pairs, it can be used to elicit efficient cleavage of long RNAs at predefined sites (Haseloff and Gerlach, 1988). This reaction has the potential to inactivate RNA molecules *in vivo*, and several such applications have appeared (Cameron and Jennings, 1994; Mathieu *et al.*, 1994; Sun *et al.*, 1994; Tang *et al.*, 1994; Zhou *et al.*, 1994; reviewed in Uhlenbeck, 1993; Marschall *et al.*, 1994). One turnover of intermolecular hammerhead cleavage can be described by (i) hybridization of the ribozyme to the substrate RNA through the formation of two helices, (ii) cleavage of the substrate and (iii) release of the 3′ and 5′ products through the dissociation of the two helices (Figure 1). Recent kinetic and thermodynamic analysis of several hammerhead cleavage reactions indicate that, as expected, the properties of the first and third steps reflect the properties of the RNA helix–coil transition (Fedor and Uhlenbeck, 1992; Hertel *et al.*, 1994).

In order for the hammerhead to be effective as a site-specific cleavage reagent, it is crucial that the ribozyme bind and cleave the correct 'target' sequence and not sequences that either contain mismatches or only part of the correct sequence. The length of the ribozyme–target helices is predicted to be critical for the specificity of cleavage (Herschlag, 1991). If the helices are too short, identical sequences present on non-target RNAs will be cleaved as efficiently as the target RNA. If the helices are too long, initial binding will be too strong and the ribozyme will cleave both correct and incorrect targets indiscriminately. In order to differentiate optimally between correct and incorrect substrates, a ribozyme requires a helix length that permits substrate dissociation to be faster than the cleavage rate. This situation allows sufficient time for the ribozyme to select correct sequences from incorrect ones prior to cleavage. A second prediction regarding specificity is that factors that increase the rate of equilibration between complexes with correct and incorrect helices can enhance specificity. RNA binding proteins that serve this role may be especially relevant for the *in vivo* use of ribozymes as site-specific cleavage agents (Tsuchihashi *et al.*, 1993; Bertrand and Rossi, 1994; Coetzee *et al.*, 1994; Herschlag *et al.*, 1994).

Here, the above expectations are tested experimentally and confirmed by using a previously well-characterized hammerhead (HH16, Figure 2) to study the cleavage of a series of truncated and mismatched substrates *in vitro*. HH16 contains two 8 bp helices and therefore cleaves a 17 nucleotide target sequence, a size commonly used in gene inactivation experiments. In addition, prior results showing that the p7 nucleocapsid protein from human immunodeficiency virus (HIV)-1 (NC) enhances specificity against a mismatched substrate are extended (Tsuchihashi *et al.*, 1993). By using a mixture of substrates generated by alkaline hydrolysis of 5′-^{32}P-labeled full-length substrate, quantitative kinetic data on a series of truncated substrate were conveniently obtained in a single experiment. The shorter substrates may be considered as models for longer substrates with limited regions of homology. This approach is analogus to that used to obtain the boundary of protein binding sites on RNA (Carey *et al.*, 1983), the sequences required for ribozyme activity

(Forster and Symons, 1987b) or the antisense RNA of optimal activity (Rittner *et al.*, 1993).

Results and discussion

Cleavage of truncated substrates of HH16

The cleavage properties of the hammerhead used in this work have been characterized extensively (Hertel *et al.*, 1994). A 38 nucleotide ribozyme (E) annealed to a 17 nucleotide full-length substrate (S) results in cleavage to give a nine nucleotide 5′ product (P1) and an eight nucleotide 3′ product (P2) (Figure 2). In order to evaluate the cleavage properties of truncated substrates, 5′-^{32}P-labeled S was subjected to partial alkaline hydrolysis. As shown in Figure 3A, lane 0, the alkaline hydrolysis conditions were adjusted so that the majority (~80%) of the RNA was full length and each shorter substrate contained 1–2% of the total radioactivity. This mixture of oligonucleotides contains eight labeled RNAs of length greater than P1 that potentially can be substrates for the ribozymes (see Figure 2 legend for nomenclature). In the experiment shown in Figure 3A, a very low concentration (~1 nM total oligonucleotide) of this mixture was combined with 500 nM ribozyme and the kinetics of cleavage were monitored on a polyacrylamide gel. Over the time course shown, nearly all of each potential substrate, with the exception of P1-G, was cleaved to form P1. The high ribozyme concentration ensures that substrate binding will be fast, and the excess of ribozyme over substrate ensures that each labeled substrate will bind independently of the other substrates and the unlabeled 3′ fragments present in the mixture. By performing radioanalytical scanning of the gel, each band was quantitated and the cleavage rate of each substrate determined. The data for the P1-GU oligonucleotide is shown in Figure 3B (closed circles) and the rate constants for each of the truncated species are given in Table I. Variation in the ribozyme concentration from 200 to 5000 nM had no significant effect on the rate of cleavage of any of the substrates, indicating that saturation was achieved. The rate constants in Table I therefore represent k_2, the rate of the chemical cleavage step.

To confirm that the rate constants obtained from the mixture accurately reflect the rate of chemical cleavage,

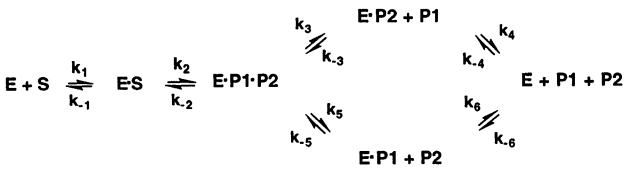

Fig. 1. Minimal kinetic scheme for hammerhead ribozyme cleavage.

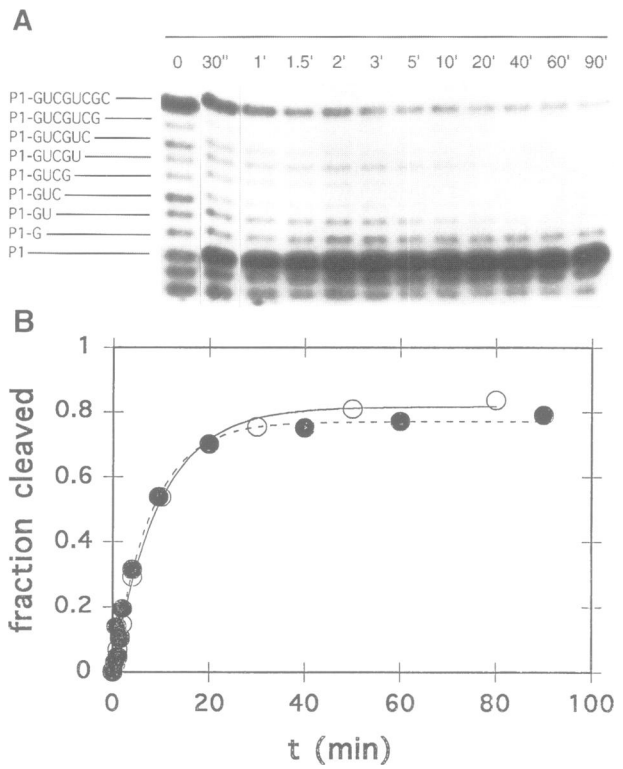

Fig. 3. Cleavage of a [5′-^{32}P]substrate mixture generated by alkaline hydrolysis. (**A**) Autoradiogram of the polyacrylamide gel showing the disappearance of each substrate band as a function of time. The lower portion of the gel showing oligomers shorter than P1 has been removed. (**B**) Quantitation of the data in (A) for P1-GU (●) and for an identical reaction with purified P·GU (○). The data are fit to k_{obs} = 0.12/min, extent = 0.78 for (●) and k_{obs} = 0.11/min, extent = 0.81 for (○).

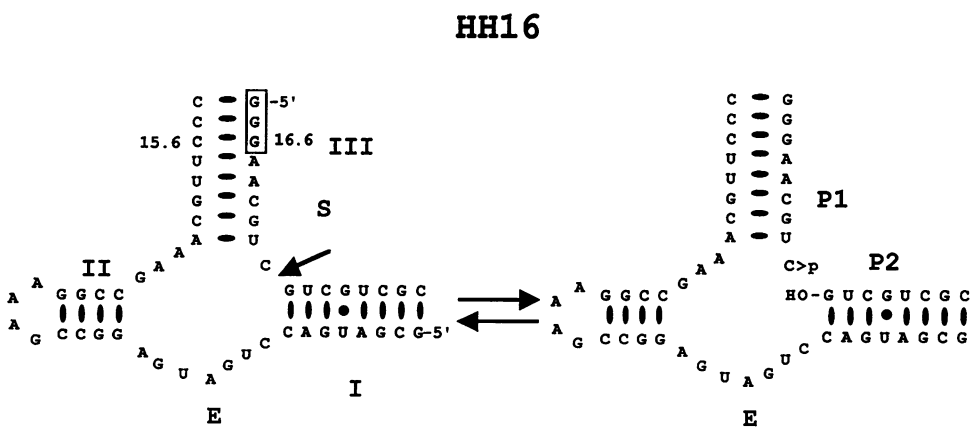

Fig. 2. Hammerhead 16 (HH16) consisting of ribozyme (E) and substrate (S). The arrow indicates the site of cleavage to give the 5′ product P1 and the 3′ product P2. DS is a truncated substrate missing the three 5′-terminal G residues (boxed). MS is a mismatched substrate where G16.6 is mutated to a U residue. P1-X corresponds to an oligonucleotide with the 5′ sequence of P1 and additional bases specified. For example, P1-GUC, MP1-GUC and DP1-GUC are S, MS and DS that have had five nucleotides removed from their 3′ terminus, respectively.

the cleavage rates of three of the oligonucleotides present in the mixture (S, P1-GUC and P1-GU) were tested individually. The data for P1-GU are shown in Figure 3B (open circles) and the rate constants determined for it and the other two oligomers were included in Table I. In all three cases, the rate constant obtained with individual oligonucleotides agrees well with the rate determined from the mixture, supporting the view that the ensemble method can be used to obtain reliable rate constants.

Five of the eight potential substrates in the hydrolysis mixture have values of k_2 close to 1/min, which is identical to the value previously obtained for HH16 (Hertel *et al.*, 1994) and for numerous other hammerheads at this pH and magnesium concentration (Uhlenbeck, 1987; Fedor and Uhlenbeck, 1990, 1992; Dahm and Uhlenbeck, 1991; Perreault *et al.*, 1991; Pieken *et al.*, 1991). Thus, these five truncated substrates combine with the ribozyme to form hammerheads that are catalytically normal. The lower rate of cleavage of P1-GU (k_2 = 0.12/min) and the very low rate for P1-G (not measured in this work) appear to arise from incomplete formation of helix I (K.Hertel, A.Perucchi, O.C.Uhlenbeck and D.Herschlag, in preparation).

The slow cleavage rate observed for P1-GUCGU (k_2 = 0.1/min) is quite surprising, especially in light of the fact that the substrates one nucleotide shorter and longer show near normal values of k_2. A possible explanation for this observation is that this substrate can form an alternative, non-productive E·S complex that rapidly exchanges with the active hammerhead. A reasonable hypothesis for such a non-productive E·S complex is that the 3′-terminal CGUCGU sequence of this truncated substrate can form a 6 bp helix with the 5′ end of the ribozyme instead of the normal helix I which would only contain 5 bp (Figure 4). The alternative helix has a calculated free energy of –9.4 kcal/mol that is substantially greater than that calculated for helix I (ΔG = –7.7 kcal/mol). Although the internal loop connecting helix I and helix III has a different number of nucleotides in the two conformations, the free energy increment for changes in the size of large loops is small (Freier *et al.*, 1986). Thus, the alternative, inactive conformation is expected to be thermodynamically favored by about 15 to 1 due to its more stable helix I. It is likely that the two structures will be in rapid exchange, since dissociation rate constants of both helices are expected to exceed 10/min based on the dissociation rates of model duplexes of comparable lengths (Turner *et al.*, 1990). Thus, the observed 10-fold slower cleavage rate agrees well with the calculated 15-fold greater stability of the alternative conformation. When the substrate is either one nucleotide longer (P1-GUCGUC) or shorter (P1-GUCG), the calculated stabilities of the alternative helix and helix I are nearly the same, suggesting that the active and inactive species will be present in similar concentrations. This is consistent with the k_2 values for PI-GUCGUC and PI-GUCG being about one half the normal rate. In the case of PI-GUC and the longer substrates, the calculated stability of the alternative pairing is much less than helix I and, as expected, a normal k_2 is observed. These results provide another example of how alternative RNA conformations can complicate the analysis of ribozyme reactions (Herschlag, 1995; Uhlenbeck, 1995).

Table I. Cleavage rates for 3′-truncated substrates

Substrate	Length	k_2 (min^{-1})[a]	k_{cat}/K_m ($\times 10^7$ M^{-1} min^{-1})[a]	Specificity[b]
P1-GUCGUCGC	17	1.2 (1.0)	1.2 (1.5)	(1)
P1-GUCGUCG	16	1.0	1.0	1.2
P1-GUCGUC	15	0.7	0.8	1.5
P1-GUCGU	14	0.1	0.5	2.4
P1-GUCG	13	0.5	1.4	0.9
P1-GUC	12	1.1 (1.0)	1.5	0.8
P1-GU	11	0.12 (0.11)	1.3 (2.0)	0.9

[a]Values in parentheses were determined with pure oligonucleotide.
[b]Specificity defined by the k_{cat}/K_m of the full-length matched substrate, S, divided by the k_{cat}/K_m of each substrate. A number >1 means that the substrate is discriminated against with respect to S.

Specificity of HH16 for shorter substrates

The ability of an enzyme to discriminate between two substrates A and B is determined by their relative second order rate constants or k_{cat}/K_m values (Scheme I; Fersht, 1975).

Scheme I

$$E + \begin{cases} S_A \xrightarrow{(k_{cat}/K_m)_A} P_A \\ S_B \xrightarrow{(k_{cat}/K_m)_B} P_B \end{cases}$$

The specificity of the enzyme for the two substrates is then defined as $(k_{cat}/K_m)_A/(k_{cat}/K_m)_B$. The values of k_{cat}/K_m for the truncated substrates were obtained by determin-

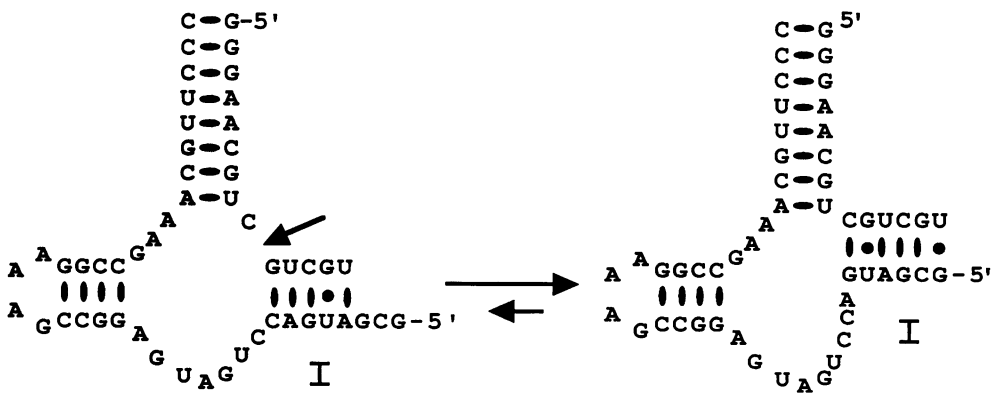

Fig. 4. Active (left) and inactive (right) alternative conformations for the hammerhead with bound P1-GUCGU substrate.

ing the rate of cleavage in substrate mixtures with varying ribozyme concentrations (Table I). k_{cat}/K_m values of two individual oligonucleotides were also tested and found to be identical to the values obtained with the mixture. Quite strikingly, all of the truncated substrates have the same k_{cat}/K_m within a factor of 2. Thus, the ribozyme does not distinguish between a full-length target that can form 16 base pairs and alternative truncated targets that can form as few as 10 base pairs, despite the fact that the shorter targets are expected to bind much less tightly. In other words, the 13.2 kcal/mol predicted difference in the free energy of binding between the full-length target and P1-GU has no effect on their relative rates of cleavage.

This extraordinary lack of specificity of the ribozyme for shorter substrates is a consequence of the fact that each forms a stable complex through helix III. As a result, each substrate is expected to dissociate from the ribozyme at a rate slower than 0.1/min, the observed dissociation rate of P1, as each substrate has minimally the same base pairing as P1 (Hertel *et al.*, 1994). Since each substrate is cleaved at ~1/min subsequent to binding, each is cleaved before it can dissociate ($k_2 > k_{-1}$). This means that under subsaturating conditions, the cleavage rate will reflect k_1, the rate of formation of the ribozyme–substrate complex. Since k_1 for hammerheads primarily reflects the rate of RNA duplex formation (Fedor and Uhlenbeck, 1992; Hertel *et al.*, 1994), it is expected to be largely insensitive to helix length.

If the presence of a stable helix III is responsible for the poor specificity of the ribozyme to the 3′-truncated substrates, one would expect that discrimination could be improved by shortening helix III to reduce its stability. This was done by preparing a substrate DS where three G residues were removed from the 5′ end of S. The rate of cleavage of DS with saturating (200–500 nM) ribozyme was determined to be 1/min, indicating that the removal of the G residues did not alter the intrinsic reactivity of the ribozyme–substrate complex. However, removal of the three G residues reduces the calculated free energy of helix III from –15.1 kcal/mol to –5.7 kcal/mol (Freier *et al.*, 1986). If the association rates are not altered, this 9.4 kcal/mol reduction in free energy means that DS is expected to dissociate from the ribozyme 8×10^6-fold faster than S. Since the dissociation rate constant of S has been estimated to be extremely slow ($k_{-1} = 1.5\times10^{-10}$/min) (Hertel *et al.*, 1994), the resulting calculated dissociation rate of DS (1.2×10^{-3}/min) is still much slower than its cleavage rate. However, one would expect that at least some of the 3′-truncated substrates derived from DS will dissociate from the ribozyme faster than the cleavage rate. These substrates would then be expected to have lower values of k_{cat}/K_m.

These expectations were confirmed by preparing a partial alkaline hydrolysis mixture of DS and determining the rates of cleavage of each truncated substrate at several enzyme concentrations. The values of k_{cat}/K_m of the truncated forms of DS are given in Table II. The 14 nucleotide DS and the first truncated form, DP1-GUCGUCG, show values of k_{cat}/K_m that are quite close to that found for S. However, k_{cat}/K_m values of the next two truncated forms, DP1-GUCGUC and DP1-GUCGU, are substantially slower, and smaller oligomers show no detectable cleavage even at long incubation times. These

Table II. Cleavage properties of shorter substrates

Substrate	k_{cat}/K_m ($\times10^7$ M^{-1} min^{-1})	Specificity[a]
DP1-GUCGUCGC	2.0	0.6
DP1-GUCGUCG	1.5	0.8
DP1-GUCGUC	0.019	63
DP1-GUCGU	~0.003	~400
DP1-GUCG	–[b]	–

[a]Specificity is defined as in Table I.
[b]No cleavage after 19 h with 20 nM E.

experimental results are in very good quantitative agreement with the calculated lower affinities of the truncated DS substrates. The removal of one, two or three residues from the 3′ end of DS is calculated to change the free energy of DS binding by +3.6, +5.7 or +8.3 kcal/mol, respectively (Freier *et al.*, 1986). If no change in the association rates occurs, the dissociation rate constants for these truncated substrates are expected to be faster than DS by a factor of 5×10^2, 2×10^4 and 1×10^6, respectively. Using the above estimate of the dissociation rate for DS of 1.2×10^{-3}/min, the rate constants for dissociation are calculated to be 0.5, 20 and 1600/min, respectively. Thus, the second and third truncated forms (DP1-GUCGUC and DP1-GUCGU) are expected to have dissociation rates well in excess of the cleavage rate, which would reduce their k_{cat}/K_m values. Indeed, this is the observed behavior.

Specificity of HH16 for mismatched substrates

To evaluate the ability of the ribozyme to discriminate against a mismatched substrate, position G16.6 in the substrate was changed to a U, creating a C·U mismatch in helix III. Since the mismatch is well away from the catalytic core of the hammerhead, it is expected to only affect the substrate binding affinity and not the catalytic rate of cleavage (Werner and Uhlenbeck, 1995). The cleavage rate constant of bound G16.6U substrate, termed MS, was determined to be 1/min, confirming the expectation that the intrinsic activity of the hammerhead was not altered by the mutation. While free energy penalties for individual single base mismatches in RNA helices have not yet been explored systematically, one would expect that the free energy of helix III would be increased by 2–4 kcal/mol as a result of the introduction of the G16.6U mutation (S.Freier, personal communication).

The cleavage rates for the series of 3′-truncated derivatives of MS prepared by partial alkaline hydrolysis were determined at several ribozyme concentrations. Figure 5 shows the gels, comparing the cleavage kinetics of MS with that of S at 40 nM ribozyme, and Table III gives the k_{cat}/K_m values for the 3′-truncated MS substrates. MS and its larger derivatives have k_{cat}/K_m values nearly identical to their fully matched counterparts. Thus, for these helix lengths, the ribozyme shows no specificity for cleavage of the matched substrate over the mismatched substrate. Thus, even with stem III weakened by the mismatch, the longer MS substrates bind with sufficient affinity such that dissociation is slower than cleavage. k_{cat}/K_m, therefore, reflects the rate of binding and, since a single mismatch will not greatly change the rate constant of helix formation (Gralla and Crothers, 1973), the longer mismatched oligo-

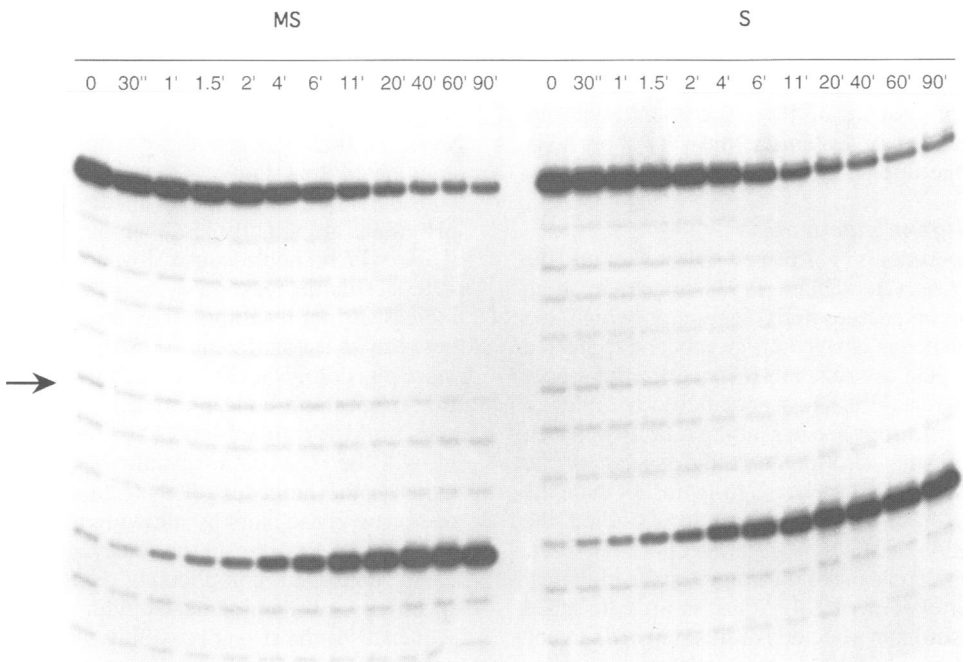

Fig. 5. Kinetics of cleavage of mismatched (MS) and matched (S) substrate mixtures generated by alkaline hydrolysis. Total substrate concentration was 0.2 nM and ribozyme concentration was 40 nM in both cases. The arrow indicates the position of MP1-GUC oligonucleotide.

mers will have cleavage properties similar to their matched counterparts.

Two truncated derivatives of MS show reduced values of k_{cat}/K_m. MP1-GUC is 6-fold slower and MP1-GU is 1000-fold slower than the full-length S substrate. As seen in Table I, the corresponding truncated derivatives of S show fast k_{cat}/K_m values. The mismatch in stem III presumably destabilizes MP1-GUC and MP1-GU sufficiently such that they frequently dissociate before cleavage, while the corresponding matched substrates do not. The slower rate of the chemical cleavage step for P1-GU is also expected to aid in discriminating against MP1-GU by allowing more time for dissociation prior to cleavage. Thus, the ribozyme was only effective in discriminating the mismatched from the matched target when the length of complementary sequence was shortened by six residues, resulting in a loss in calculated substrate binding energy of nearly 13 kcal/mol. This need to reduce substrate binding energy to achieve discrimination by an RNA enzyme reflects the high stability of nucleic acid helices.

Effect of HIV nucleocapsid protein on specificity

The cleavage rates for the substrate mixtures of S and MS were determined at several subsaturating ribozyme concentrations in the presence of either 200 or 400 nM nucleocapsid protein. Previous work indicated that these concentrations were in a range where the hammerhead helix stability was affected without greatly disrupting the catalytic core (Tsuchihashi *et al.*, 1993). Table IV gives the ratio of k_{cat}/K_m values for each truncated matched and mismatched substrate determined in the absence and in the presence of protein. In agreement with Table III, in the absence of protein, very little specificity was observed for the longer oligomers and only some specificity was seen for the 12mer. With increasing concentrations of nucleocapsid protein, however, there is specificity against some of the longer substrates. At 200 nM protein, there is significant specificity against the mismatched 13mer substrate (data not shown) and, at 400 nM protein, clear discrimination is observed even against the 15mer. Thus, the substrate length where specificity is observed is

Table III. Cleavage properties of mismatched substrates

Substrate	Length	k_{cat}/K_m ($\times 10^7$ M^{-1} min^{-1})	Specificity[a]
MP1-GUCGUCGC	17	1.2	1.0
MP1-GUCGUCG	16	0.8	1.5
MP1-GUCGUC	15	0.8	1.5
MP1-GUCGU	14	0.6	2.0
MP1-GUCG	13	0.4	3.0
MP1-GUC	12	0.2	6.0
MP1-GU	11	0.0012	1000

[a]Specificity defined as in Table I.

Table IV. Effect of nucleocapsid protein on specificity between S and MS

Substrate (P1- and MP1-)	Length	Specificity[a]	Specificity with 400 nM protein
-GUCGUCGU	17	2	2
-GUCGUCG	16	2	2
-GUCGUC	15	2	6
-GUCGU	14	_[b]	_[b]
-GUCG	13	3	50
-GUC	12	11	>100

[a]Specificity defined as k_{cat}/K_m of each 3′-truncated variant of the matched substrate divided by k_{cat}/K_m of the corresponding 3′-truncated variant of the mismatched substrate (MS). This definition is different from that in Tables I and II in order to standardize length effects.
[b]HH16 concentrations used were saturating for this substrate so k_{cat}/K_m values could not be determined.

increased by three or four residues. The value of k_{cat}/K_m for the shortest active matched substrate (P1-GU) is so greatly reduced at 400 nM protein that no cleavage was observed in the time scale of the experiment. For this substrate, the protein may destabilize the very short helix I such that cleavage does not occur.

Implications for gene inactivation

The *in vitro* experiments performed here support the theoretical prediction (Herschlag, 1991) that there is an upper limit of hammerhead helix length at which the correct target sequence is cleaved efficiently and truncated or mismatched targets are not. HH16 exceeds this upper limit and, therefore, exhibits low specificity. In order to exploit optimally the difference in binding affinity between the matched and mismatched targets, the dissociation rate of the matched target should be slightly faster than the chemical cleavage step. Under the conditions studied, the substrate dissociation of HH16 is ~10 orders of magnitude slower than the rate constant of cleavage for the chemical step of 1/min (Hertel *et al.*, 1994). Thus, in order to make HH16 able to discriminate truncated or mismatched targets, the dissociation rate must be increased by substantially reducing the lengths of helices I and/or III. This was demonstrated by the ability of the ribozyme to cleave DP1-GUCGUCG 80-fold faster than a substrate one nucleotide shorter (Table II) and by its ability to cleave P1-GU ~1000-fold faster than the corresponding mismatched target, MP1-GU (Table III). Thus, the hammerhead ribozyme has an intrinsic ability to effectively discriminate between single base changes. It remains to be determined if there are helix lengths that are both short enough to allow discrimination against single base changes and long enough to recognize and cleave a unique RNA target. In addition, the degree to which cleavage of non-target RNAs is detrimental will presumably vary in individual cases.

While the upper limit of target helix length needed for high specificity is between 10 and 12 bp for HH16 under the reaction conditions studied, this length will vary depending upon a number of intrinsic and extrinsic factors. One important factor is the actual length and sequence of the individual hammerhead arms. While the rate of chemical cleavage has been found to be nearly independent of the sequence of the arm (Fedor and Uhlenbeck, 1990), the affinity of the ribozyme for its target is expected to be sequence dependent. As a result, the helix lengths at which the dissociation rate exceeds the cleavage rate will vary. For example, variants of HH16 with helices that contain more A·U pairs, and hence have lower stability, would be expected to exhibit an increase in the upper limit of target helix length that shows high specificity. It has been proposed (Hertel *et al.*, 1994) that the free energy of formation of the ribozyme–substrate complex can be expressed as the sum of a constant contribution from the hammerhead core and a variable contribution from the helices that can be calculated from the known parameters of RNA helix stability (Freier *et al.*, 1986). If this relationship is true, it should be possible to predict accurately the affinity of a ribozyme for its substrate and, hence, the upper limit of helix length that permits high specificity. Experimental verification of this proposal is, therefore, of considerable interest.

Since the extrinsic variables of pH, temperature and divalent ion concentration all affect the rate of chemical cleavage of the hammerhead, the upper limit of helix length that gives high specificity is expected to be longer inside cells than the 10–12 bp found for HH16 in this work. Although no good measurement is available, it is anticipated that the rate of chemical cleavage will be considerably less than 1/min inside most cells. Intracellular pH values are slightly less than the pH of 7.5 used in this work, which should cause slower cleavage (Dahm *et al.*, 1993). This decrease in k_2 will be roughly offset by the increase in k_2 resulting from the temperature difference between the experiments presented here (25°C) and the more physiological (37°C). The relatively low intracellular free magnesium concentration of 0.5 mM for eukaryotes and 2 mM for prokaryotes (Maguire, 1990) suggests that k_2 will be <0.1/min (Dahm *et al.*, 1993). This may increase the helix length that allows high specificity by one or two base pairs by allowing more time for substrate dissociation to occur before cleavage.

An important complicating factor in evaluating the specificity of hammerheads with different helical arm sequences is the strong possibility that either the ribozyme or the target RNA can adopt alternative conformations that may greatly decrease their mutual binding affinity. This problem is especially relevant to gene inactivation experiments where the target sequence is embedded in a longer RNA and, therefore, has an increased potential of forming higher order structures. If the alternative, non-target sites are more accessible for hybridization than the target, the optimal specificity will be less than that observed in experiments with short, unstructured targets such as those presented here. If the target site is more accessible for hybridization than any non-target, the specificity will be greater. This clearly emphasizes the need to locate sites in mRNA targets that are accessible for hybridization.

Further, it is necessary to assess the accessibility of RNA targets and ribozymes *in vivo* rather than *in vitro* for gene inactivation. RNA binding proteins have been shown to have profound effects on RNA accessibility and on ribozyme function *in vitro* (Table IV) (Tsuchihashi *et al.*, 1993; Bertrand and Rossi, 1994; Coetzee *et al.*, 1994; Herschlag *et al.*, 1994; Muller *et al.*, 1994). These proteins appear to act by increasing the rate of duplex unwinding, thereby enhancing turnover when the release of the cleaved RNA is rate-limiting and enhancing specificity by allowing the ribozyme to sample an array of potential RNA substrates prior to cleavage. On the other hand, RNA binding proteins can also mask RNA sites and inactivate ribozymes (Herschlag *et al.*, 1994; Herschlag, 1995). Direct investigations will be required to understand the interplay of these stimulatory and inhibitory effects *in vivo*.

Materials and methods

RNA synthesis

Substrate oligonucleotides were synthesized chemically and ribozymes were synthesized by *in vitro* transcription as described previously (Hertel *et al.*, 1994). A mixture of 5′-^{32}P-labeled oligonucleotides was prepared by partial alkaline hydrolysis of the corresponding labeled substrate. Sixteen μl of 1 nM 5′-^{32}P-labeled RNA in water was mixed with 4 μl of 1 M NaOH and incubated at 25°C for 5 min. The reaction was terminated by the addition of 8 μl of 0.5 M Tris–HCl and kept frozen until used.

Cleavage reactions

All reactions were conducted at 25°C in 50 mM Tris–HCl, pH 7.5 and 10 mM $MgCl_2$, essentially as described previously (Hertel *et al.*, 1994). In order to disrupt potential aggregates formed during storage, ribozyme, substrate or substrate mixtures were heated to 95°C for 1.5 min in buffer without $MgCl_2$ and then allowed to cool to 25°C. $MgCl_2$ was then added to a final concentration of 10 mM. Unless stated otherwise, reactions were initiated by combining various concentrations of ribozyme (5 nM–1 μM) in 20 μl reaction buffer with 20 μl of ~1 nM radiolabeled substrate or substrate mixture in reaction buffer. In some cases, ribozyme and substrate (or substrate mixture) were heated and cooled together in buffer and the reaction was initiated by the addition of $MgCl_2$ to 10 mM. At saturating ribozyme concentrations, the latter protocol gave slightly higher extents of cleavage, but very similar rate constants. In both protocols, aliquots (usually 3 μl) were withdrawn at appropriate times and quenched with 10 μl of 8 M urea, 50 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol. During long reactions, solutions were centrifuged periodically to minimize the effect of evaporation. Reactions were analyzed on 20% polyacrylamide–7 M urea gels and the radioactive bands were located and quantitated with a radioanalytical scanner (Molecular Dynamics). Pseudo-first order rate constants were determined by measuring the decrease in relative radioactivity over time for reactions with different concentrations of ribozyme present. The relative radioactivity at each time point was calculated by computing the fraction of disintegrations in the substrate band over the sum of disintegrations of all substrate and product bands within each lane. Rate constants for each substrate were determined from a non-linear least-squares fit (KaleidaGraph, Ablebeck Software) of the data to an expression describing first order accumulation of product. Each rate constant is an average of at least two determinations. Rates determined on different days with different dilutions of stock solutions varied by <2-fold.

Cleavage reactions in the presence of the p7 nucleocapsid (NC) protein were performed essentially as described above. NC protein was purified as described previously (Tsuchihashi and Brown, 1994). NC (200–400 nM) or buffer was added to the ribozyme solution up to 1 min prior to the addition of the substrate solution. Uncertainties in the specificity ratios were estimated to be ~2-fold from independent experiments.

The kinetic parameter k_2 represents the rate constant for the ribozyme–substrate complex (Figure 1). For the measurements herein with saturating ribozyme, there is no contribution from product dissociation as the reactions were single-turnover and the reverse reaction (k_{-2}) is negligible (Hertel *et al.*, 1994). The kinetic parameter k_{cat}/K_m represents the second order reaction of free ribozyme and free substrate (Fersht, 1975). For reactions with an excess of ribozyme over substrate, two criteria are diagnostic for k_{cat}/K_m or subsaturating conditions: (i) first order disappearance of substrate, which indicates that the reaction is first order in substrate, and (ii) a linear increase in k_{obs} with increasing ribozyme concentration, which indicates that the reaction is first order in ribozyme. Both conditions were met and values of k_{cat}/K_m were determined from slopes of plots of k_{obs} versus ribozyme concentration, with ribozyme concentration limited to the linear range.

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