Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 6977–6981, July 1994
Biochemistry

Kinetic characterization of intramolecular and intermolecular hammerhead RNAs with stem II deletions

DAVID M. LONG AND OLKE C. UHLENBECK*

Department of Chemistry and Biochemistry, Campus Box 215, University of Colorado, Boulder, CO 80309-0215

Contributed by Olke C. Uhlenbeck, April 11, 1994

ABSTRACT **A method is described to obtain intramolecular cleavage rates for the hammerhead ribozyme during *in vitro* transcription. By avoiding RNA purification and renaturation, the potential for formation of inactive conformations of the RNA is minimized. By showing that an intramolecular hammerhead and a closely related intermolecular hammerhead cleave at the same rate under a given set of conditions, we confirm that both reactions probably have the same rate-limiting step. An *in vitro* selection strategy was used to isolate active hammerheads from a library of molecules where six randomized nucleotides replaced stem–loop II. The sequence and number of nucleotides which replace stem–loop II have large effects on hammerhead cleavage activity. The relative activities of three sequences selected from the intramolecular library are the same when the sequences are transferred into an intermolecular hammerhead background.**

The hammerhead is an RNA structural motif of about 50 nucleotides that is embedded within the sequences of certain plant pathogenic RNAs and is responsible for self-cleavage activity (1–3). Intramolecular hammerhead cleavage has been studied by isolating uncleaved RNA from *in vitro* transcription reactions and introducing it into the desired reaction buffer (4, 5). Because hammerheads often self-cleave quite efficiently during transcription, it is possible that the small fraction of uncleaved RNA might contain mutations as a result of misincorporation and thus may not have the same cleavage properties as the presumed RNA sequence. In addition, when the RNA is introduced into the reaction buffer, a rate-limiting conformational change may precede cleavage. To avoid these problems, a common approach to study hammerhead cleavage under defined conditions has been to divide the hammerhead into separate RNAs and combine them to initiate the reaction (6, 7). However, because this introduces an additional assembly step in the reaction pathway, it is critical to show that closely related intramolecular and intermolecular hammerheads cleave at the same rate. Indeed, based on the rapid cleavage of an intramolecular hammerhead (8), Forster and Symons (9) have suggested that intermolecular hammerhead cleavage rates may reflect formation of the active intermolecular complex and not the chemical step of the reaction.

To compare intramolecular and intermolecular hammerhead cleavage rates under identical conditions, a method to measure intramolecular cleavage rates in transcription reactions has been developed. To be certain that the method is effective with a variety of hammerheads, a library of hammerheads was prepared where six randomized nucleotides replaced stem–loop II. It is known that the sequence of stem–loop II is not strictly conserved (2, 9, 10) and replacement of stem–loop II with shorter sequences reduces but does not abolish cleavage activity (10–13). By selecting active molecules from the library, hammerheads with a range of cleavage activities were isolated. Cleavage rates were then measured in both intramolecular and intermolecular backgrounds to directly address the relationship between the two types of hammerhead cleavage.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

RNA Synthesis. RNAs were synthesized by transcription of partially duplex synthetic DNA templates by T7 RNA polymerase (14). In cases where cloned hammerheads were transcribed, plasmid DNA was linearized with restriction endonuclease *Bam*HI, which resulted in 5 nucleotides (GGAUC) on the 3′ end in addition to those shown in Fig. 1. Randomized positions in the RNA were obtained by using mixtures of all four deoxynucleoside triphosphates (dNTPs), adjusted for different phosphoramidite reactivities, at the corresponding steps in DNA template synthesis. Transcription reaction mixtures (10 μl) contained, unless otherwise stated: 200 nM DNA template, 40 mM Tris·HCl (pH 8.1), 11 mM $MgCl_2$, 10 mM spermidine, 50 mM dithiothreitol, bovine serum albumin (0.5 mg/ml), 0.1% Triton X-100, T7 RNA polymerase (0.1 mg/ml), and 2 mM each NTP, with 10 μCi of [α-^{32}P]CTP (1 μCi = 37 kBq). Incubation was at 37°C. The 12-nucleotide substrate RNA used in intermolecular cleavage reactions was chemically synthesized by solid-phase phosphoramidite chemistry (15) and standard deprotection and PAGE purification procedures. RNA was 5′ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol).

Selection Procedure. Reverse transcription and PCR were used to amplify those sequences in the library that had cleaved to form the expected 45-nucleotide product during transcription. After 30 min at 37°C, reactions were quenched with a 5-fold excess of 9 M urea/50 mM EDTA/0.04% bromophenol blue/0.04% xylene cyanol and fractionated by electrophoresis in 15% polyacrylamide/7 M urea gels. The band corresponding to cleaved RNA was located by autoradiography, excised, and soaked for 2 hr in 0.4 M ammonium acetate (pH 5.4). RNA was ethanol precipitated and reverse transcribed in 20 μl of 1 μM DNA primer (5′-GGCGATGGATCCGCGACGACGACGTTCTCTCG-3′)/20 mM Tris·HCl, pH 8.3/40 mM KCl/6.5 mM $MgCl_2$/0.2 mM dNTPs with ≈5 units of avian myeloblastosis virus reverse transcriptase for 30 min at 37°C. The eight nucleotides lost upon hammerhead cleavage were restored with this primer. The reaction products were ethanol precipitated and suspended in 20 μl of 1 mM Tris·HCl, pH 7.5/0.1 mM EDTA. Four microliters of the cDNA was used in 40-μl PCR mixtures that contained 20 mM Tris·HCl (pH 8.3), 50 mM NaCl, 0.1% gelatin, 1 mM $MgCl_2$, 0.2 mM dNTPs, the reverse transcriptase primer, and a second primer (5′-GCGCTAGAATTC-TATAACGACTCACTATAGCGATGA-3′) containing the T7 RNA polymerase promoter (underlined sequence) and were incubated at 95°C (30 sec), 55°C (10 sec), and 72°C (2 min) for from 10 to 12 cycles. In the next round of the

*To whom reprint requests should be addressed.

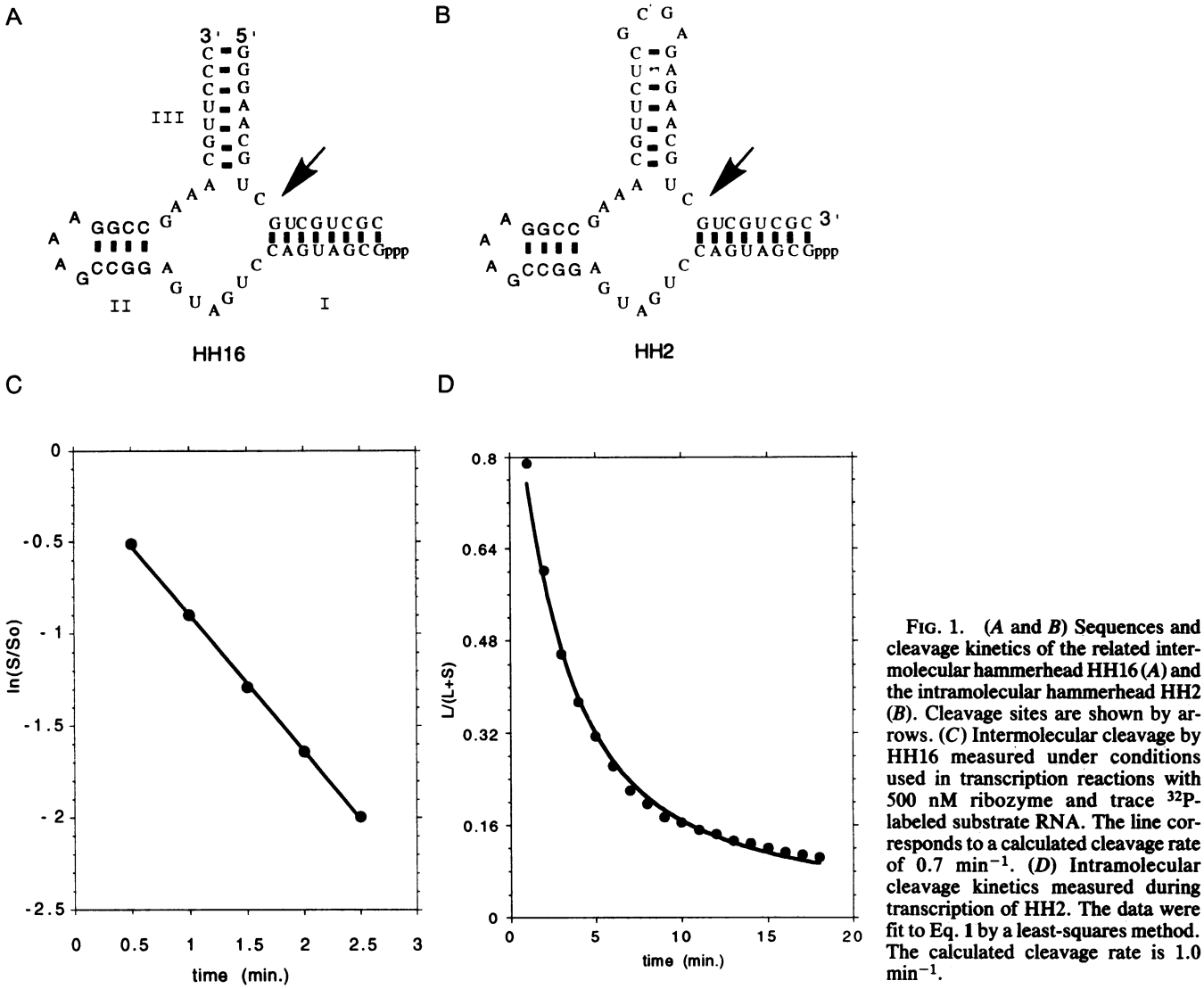

FIG. 1. (*A* and *B*) Sequences and cleavage kinetics of the related intermolecular hammerhead HH16 (*A*) and the intramolecular hammerhead HH2 (*B*). Cleavage sites are shown by arrows. (*C*) Intermolecular cleavage by HH16 measured under conditions used in transcription reactions with 500 nM ribozyme and trace ^{32}P-labeled substrate RNA. The line corresponds to a calculated cleavage rate of 0.7 min^{-1}. (*D*) Intramolecular cleavage kinetics measured during transcription of HH2. The data were fit to Eq. 1 by a least-squares method. The calculated cleavage rate is 1.0 min^{-1}.

selection procedure, 9 μl of the PCR mixture was used in a 20-μl transcription mixture.

After three cycles of selection, DNA was purified by electrophoresis in a nondenaturing 15% polyacrylamide gel and cloned into the *Eco*RI and BamHI restriction sites of plasmid pUS618 (16). Colonies containing inserted PCR product were identified by hybridization and sequenced by using Sequenase 2.0 and protocols supplied by the manufacturer (United States Biochemical).

Hammerhead Cleavage Kinetics. Steady-state parameters for intermolecular hammerhead cleavage were measured as described (17). Reactions were carried out at 25°C in 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$. Reaction rates were measured by determining the extent of cleavage at various times from 20% acrylamide/7 M urea gel fractionation and quantitation with a Molecular Dynamics PhosphorImager. For determination of k_{cat} and K_m, reaction rates were measured for at least five different RNA substrate concentrations. Results were analyzed by Eadie–Hofstee plots (18).

Because intramolecular hammerheads cleave under the conditions used to transcribe these RNAs, a method was devised for measuring cleavage rates during transcription. Although the rate of accumulation of cleaved transcript depends on both the transcription and cleavage rates, the fraction of total transcribed RNA which remains uncleaved is independent of the transcription rate as long as it does not change during the time of analysis. Transcription reactions can be carried out at constant rates under a broad range of conditions, including all those described in this paper (19). Eq. 1 can be derived for the case where full-length transcript is an intermediate in a two-step reaction consisting of transcription followed by cleavage

$$\frac{L}{L+S} = \frac{1}{kt}(1 - e^{-kt}), \qquad [1]$$

where L is the concentration of full-length transcript, S is the concentration of cleaved transcript, t is time, and k is the unimolecular rate constant for cleavage. Parameters which describe kinetics of transcription cancel out in the derivation of this expression. Intramolecular cleavage rates were determined by plotting $L/(L+S)$ as a function of t and fitting the data by a least-squares method to Eq. 1.

RESULTS

To determine whether an intramolecular hammerhead and a similar intermolecular hammerhead had similar cleavage activities, the kinetic behaviors of hammerheads 16 and 2 (HH16 and HH2) were compared under identical conditions (Fig. 1). HH16, which has relatively long stems I and III that result in strong substrate binding, was chosen because its cleavage properties had been extensively characterized (20). HH2 is identical to HH16 but has a loop that closes stem III and one mutated base pair in stem III. The rate of intramolecular cleavage by HH2 was measured during transcription,

whereas the rate of intermolecular cleavage by HH16 was measured at saturating ribozyme concentration in transcription buffer without T7 RNA polymerase. As is shown in Fig. 1, the rate of HH16 cleavage is 0.7 min^{-1}. This value is somewhat less than expected for cleavage at pH 8.1 (20), but the difference can probably be accounted for by the lower free Mg^{2+} concentration in the transcription buffer. HH2 cleaves at a rate of 1.0 min^{-1}, indicating that both the intramolecular and intermolecular hammerheads have virtually the same cleavage rate. In addition, both cleavage reactions continue to near completion, indicating that the measured rates represent a majority of the hammerhead molecules in each reaction mixture. Thus, there is not a large fraction of inactive hammerheads resulting from, for example, incorrectly folded RNA.

The cleavage rates for both HH16 and HH2 were measured as a function of pH, and in both cases the logarithm of the cleavage rate was linear with respect to pH (data not shown). This behavior has been observed for another hammerhead (21) and indicates that the chemical cleavage step is rate limiting in these reactions. Cleavage of hammerheads rate-limited by RNA conformational changes is not expected to be pH sensitive.

To obtain a variety of hammerheads with different activities, a library of intramolecular hammerheads with six randomized nucleotides at the position of helix II was used (Fig. 2*A*). This library was expected to contain hammerheads with a wide range of activities because the primary structure of stem–loop II is nonessential but is known to affect hammerhead activity (10–13). The transcription reaction for the library of hammerheads (Fig. 2*B*) shows that the overall rate is much slower than HH2, but about 40% of the full-length transcript is cleaved after 50 min. While one might be tempted to conclude that as many as 1600 of the 4096 sequences are active, it is difficult to accurately estimate the number of active sequences because species in the mixture could form intermolecular complexes that could be either more or less active. Indeed, when the uncleaved RNA in Fig. 2*B* was removed from the gel and renatured, a larger fraction than would be predicted from Eq. 1 cleaved when incubated in transcription buffer a second time.

Because our primary interest was in the more active members of the library, we carried out an enrichment procedure that facilitated identification of these hammerheads. The procedure entailed isolating the 45-nucleotide cleavage product and converting it to DNA by reverse transcription and PCR. Since the primer used in reverse transcription restored the 8 nucleotides that were lost upon cleavage and the PCR primer reintroduced the T7 RNA polymerase promoter, the library could be transcribed a second time. Enrichment was confirmed by finding that the amount of cleaved RNA after 5 min of transcription increased from 12% for the original library to 18%, 21%, and 25% after successive cycles of enrichment were performed. Further enrichment was not apparent after the procedure was carried out for additional cycles.

Double-stranded cDNA isolated after the third cycle of enrichment was cloned and individual sequences were determined and tested for cleavage activity. Out of seven sequences tested, five had detectable cleavage activity. The active clones all had a pyrimidine at position 11.1, indicating that this is a requirement as previously identified by phylogenetic comparison and mutagenesis experiments (9, 10). To slow cleavage rates and make them more easily measurable, individual clones were transcribed and intramolecular cleavage rates were measured at pH 7.0. The variants have cleavage rates which vary over 3 orders of magnitude and which are arbitrarily grouped into three categories (fast, medium, and slow). Representative kinetic plots from each group are shown in Fig. 3. Of the three hexanucleotides

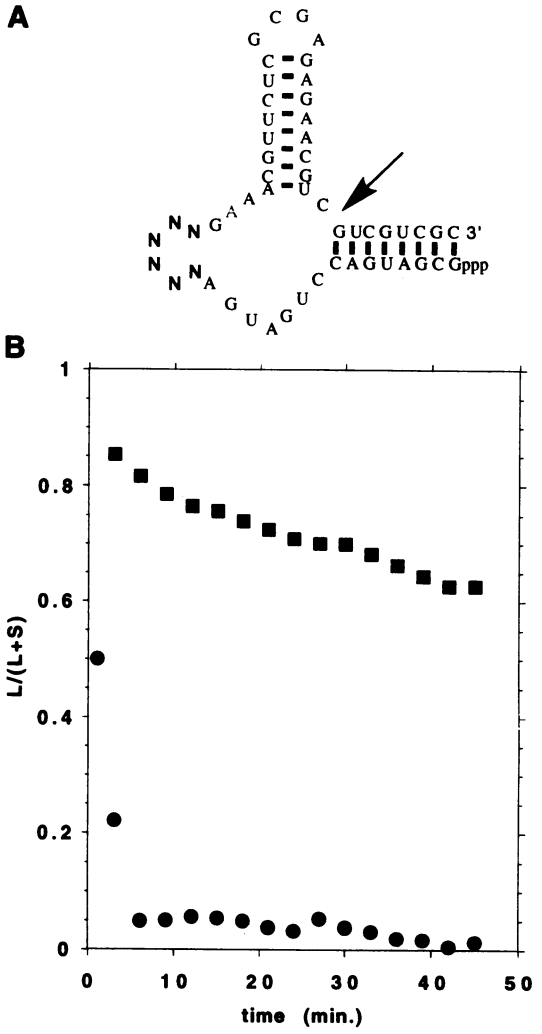

FIG. 2. Sequence and cleavage kinetics of the hammerhead library with six randomized nucleotides at stem II. (*A*) N represents a randomized position in the sequence and the expected cleavage site is shown by the arrow. (*B*) Intramolecular cleavage kinetics measured during transcription of the library (■) and HH2 (●).

shown, GUUUGC has the highest activity (10% that of the control hammerhead), UUUCUU has intermediate activity, and ACUCCC has undetectable activity (<0.001 min^{-1}).

To compare effects of helix II sequences on substrate binding and catalysis in intermolecular cleavage reactions the three representative hexanucleotides were incorporated into the HH8 background (Fig. 4). Unlike HH16, the Michaelis constant (K_m) for HH8 is equal to the equilibrium dissociation constant (K_d), and so by measuring kinetics in this background it is possible, in some cases, to estimate equilibrium binding constants. Kinetic parameters for HH8 have been measured under a variety of conditions (17, 21, 22).

Cleavage kinetics for HH8 and the three hexanucleotides in the intramolecular and the intermolecular backgrounds are compared in Table 1. Since the intramolecular and intermolecular rates were measured under different conditions, it is fortuitous that their absolute values agree. However the important point is that the relative rates for all three constructs are maintained upon transfer into the intramolecular background, indicating that for a given hexanucleotide replacement, the intramolecular and intermolecular rates are probably limited by the same process. In the intermolecular reactions both decreases in k_{cat} and increases in K_m were observed, indicating that the sequence at stem–loop II affects both substrate binding and the rate of substrate cleavage.

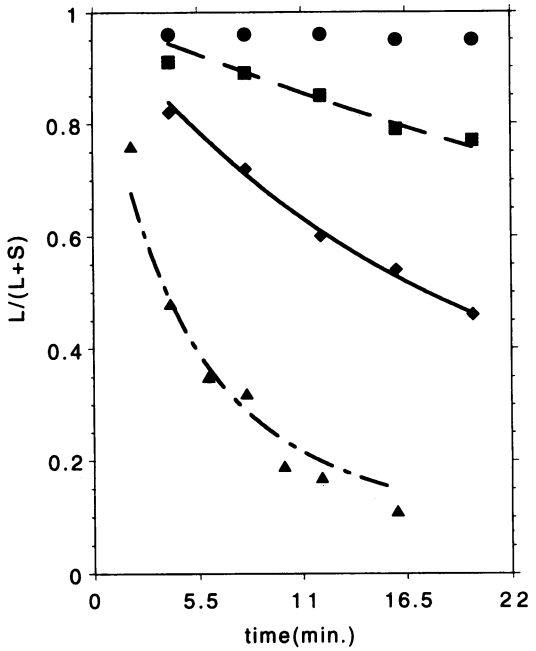

FIG. 3. Intramolecular cleavage kinetics measured during transcription at pH 7.0 for selected stem II variants (●, ACUCCC; □, UUUCUU; ◆, GUUUGC) and HH2 (▲). Cleavage rates derived from these data are in Table 1.

In addition to stem–loop II variants with hexanucleotide replacements, three intermolecular hammerheads with tetranucleotide replacements at stem–loop II were constructed and kinetically characterized (Table 2). Both hexanucleotide and tetranucleotide helix II replacements are expected to have sufficient length to replace stem–loop II. The sequence UUUU was chosen because the kinetic effects of this sequence had been determined in the context of a different intermolecular hammerhead background (12). The tetranucleotides UGAC and CUCC were chosen because the former sequence is about 10 times more effective than the latter at replacing the anticodon stem–loop in yeast phenylalanyl tRNA (23). We wanted to test whether the relative and absolute abilities of these two sequences to replace stem–loop II in the hammerhead were similar to those observed in tRNA.

Table 2 shows cleavage kinetics for hammerheads with tetranucleotide stem–loop II replacements. Tetranucleotide replacements appear to be less effective than hexanucleotide replacements at stem–loop II in the HH8 background. Table 2 does not contain values for K_m, because slow cleavage rates precluded measurement of these constants. The sequence UUUU incorporated into HH8 resulted in a 200-fold reduction in k_{cat}. As judged by cleavage kinetics the effectiveness

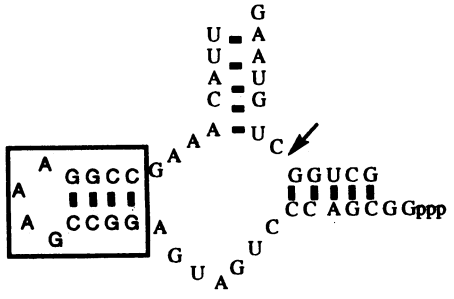

FIG. 4. Sequence of HH8. The cleavage site is marked by an arrow and residues in the box were replaced by four or six nucleotides.

Table 1. Intramolecular and intermolecular cleavage kinetics for hammerheads with hexanucleotide helix II replacements

Sequence	k_{cis},* min^{-1}	k_{cat},† min^{-1}	K_m, μM	k_{cat}/K_m, $M^{-1}\cdot min^{-1}$	Relative k_{cat}/K_m
GUUUGC	0.09	0.12	1.5	8×10^4	3×10^{-3}
UUUCUU	0.03	0.05	0.2	2.5×10^5	1×10^{-2}
ACUCCC	<0.001	<0.001	—	—	—
A^AGGCC A_GCCGG	1.0	1.5	0.04	3.8×10^7	1.0

*Intramolecular cleavage.
†Intermolecular cleavage.

of UGAC is 10-fold greater than CUCC in the HH8 background, although both sequences cause significant reductions in activity (up to 1000-fold). Thus the relative ability of the sequences UGAC and CUCC to replace stem–loops in the hammerhead and yeast phenylalanyl tRNA are the same.

DISCUSSION

We have shown that intramolecular cleavage rates can be determined during *in vitro* transcription, using a method that does not require gel purification, concentration, and renaturation of self-cleaving RNAs. The kinetic analysis applies to the general case of a two-step chemical reaction where an intermediate species produced at a constant rate undergoes unimolecular decay. Application of the method to self-cleaving RNAs requires that a plot of total transcript versus transcription time be linear.

Although Eq. 1 holds for any unimolecular decay rate, practical application of the method is limited to measurement of intramolecular cleavage rates between about 10^{-4} per minute and several per minute. When cleavage rates are fast, the method is limited by the sensitivity of detection of substrate, and when the cleavage rates are slow, the method is limited by the sensitivity of detection of product. For self-cleavage reactions limited by the chemical cleavage step, these limits can be extended somewhat by changing the pH of the transcription reaction mixture. Because other self-cleaving RNAs cleave at rates comparable to the hammerhead (3), we expect that this approach can be used for kinetic studies of these RNAs as well.

Although the self-cleaving hammerheads studied here are rate-limited by the chemical cleavage step, the methods we describe are expected to be equally useful for measuring self-cleavage kinetics that are limited by other processes—for example, changes in RNA conformation. An example of a self-cleaving RNA with rate-limiting structural change is the plus strand of Lucerne transient streak virus (2, 9, 24). This RNA can assume two distinct structures in solution, only one of which contains an active hammerhead structure. Mutants of a hammerhead derived from this RNA show different extents of cleavage during transcription (25), but whether this results from alterations in conformational properties or changes in intrinsic catalytic activity of the hammerhead is not clear. Intramolecular cleavage rates measured during transcription at different pH values should resolve this point.

Table 2. Cleavage kinetics for intermolecular hammerheads with tetranucleotide helix II replacements

Sequence	k_2, min^{-1}
UUUU	0.005
UGAC	0.012
CUCC	0.00096
A^AGGCC A_GCCGG	1.5

We have shown that our selection procedure, based on intramolecular hammerhead activity, is useful for identifying hammerheads with high intermolecular cleavage activity. The selection methods we describe could be further exploited to identify optimum helix II replacements or hammerhead mutations which preserve or alter activity in a desired way. For example, it may be possible to find mutations which increase activity when limiting Mg^{2+} is present.

Most hammerhead studies have employed intermolecular RNAs, but natural hammerheads are active as intramolecular RNAs (4, 5). Since several intramolecular hammerheads were found to cleave at rates comparable to the corresponding intermolecular hammerheads, it appears that there is no fundamental kinetic difference between the two reactions as had previously been suggested (8). Thus kinetic studies of the hammerhead ribozyme can employ either type of hammerhead, the choice dictated largely by the specific goals of each experiment.

Hammerhead activity depends on the sequence and number of bases which replace helix II. Of the helix II replacements with six or four nucleotides that were tested here, none are as active as the parent hammerheads. Of those tested, the most effective hexanucleotide replacement is GUUUGC, the first and last nucleotides of which could form a G·C base pair while the second and fifth could form a G·U wobble pair. Indeed Tuschl and Eckstein (13) concluded from mutational studies of stem–loop II that the minimal structural requirement for this part of the hammerhead was a 2-bp stem. Mutations in stem–loop II in the hammerhead background used by Tuschl and Eckstein mainly affect the k_{cat} of these intermolecular hammerhead reactions. However, we have identified stem–loop II replacements in another hammerhead background which have relatively large effects on K_m in addition to k_{cat}. Although the reason for this is not clear, further kinetic analyses of these interesting hammerheads might resolve this issue.

Our UUUU hammerhead has the same k_{cat}, when adjusted for pH and temperature, as the cleavage rate reported for UUUU in the hammerhead studied by McCall *et al.* (12). However, their control hammerhead cleaves about 100 times slower than expected for hammerhead cleavage at pH 8.0 and 37°C (K. J. Hertel and O.C.U., unpublished observations). This is probably because they employed a substrate oligonucleotide that has the potential to dimerize through formation of 10 consecutive base pairs. Indeed, evidence for substrate dimerization was clearly shown by their nondenaturing gel experiments (12). Although McCall *et al.* (12) concluded that replacing stem–loop II with a tetranucleotide affected hammerhead activity only moderately, our tetranucleotide replacement experiments and those reported by Tuschl and Eckstein (13) indicate that this type of alteration has significant consequences on hammerhead activity. Thus, these small hammerheads probably are not well suited as gene-inactivating reagents.

The results of our helix II replacement experiments show that it might be possible to identify short sequences that are useful stem–loop replacements within the contexts of different folded RNAs. The degree to which a sequence compromises or preserves activity seems to hold faithfully when that sequence is transferred from one hammerhead to another (Table 1). Stem–loop replacement experiments in tRNA have given a similar result. Of the two tetranucleotides UGAC and CUCC, the former is 10-fold more effective as a structural replacement for both helix II in the hammerhead and the anticodon stem–loop in yeast phenylalanyl tRNA (Table 2). Effective deletion of stem–loop structures in large folded RNAs could be a useful route to molecules more amenable to structural and functional studies.

It is interesting that replacement of stem–loops in tRNA with tetranucleotides can have little effect on the absolute stability of the molecule, whereas replacement in the hammerhead apparently causes a significant disruption in the stability of the active structure. A possible explanation for this is that the tRNA has a larger free energy of folding and can consequently withstand more disruptive changes than the hammerhead can. If the hammerhead has a smaller free energy of folding than the tRNA, tetranucleotide replacements may contribute unfavorably to folding, enough in some cases to prevent formation of the active structure. For this reason it is possible that stem–loop replacements in large RNAs will prove to be more successful than in small RNAs such as the hammerhead.

We thank Doug Turner for useful comments concerning the manuscript. This work was sponsored by grants from the National Institutes of Health (O.C.U.). D.M.L. is supported by a postdoctoral fellowship from the National Institutes of Health (GM14471).

1. Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R. & Bruening, G. (1986) *Science* **231**, 1577–1580.
2. Forster, A. C. & Symons, R. H. (1987) *Cell* **49**, 211–220.
3. Long, D. M. & Uhlenbeck, O. C. (1993) *FASEB J.* **7**, 25–30.
4. Hutchins, C. J., Rathjen, P. D., Forster, A. C. & Symons, R. H. (1986) *Nucleic Acids Res.* **14**, 3627–3640.
5. Forster, A. C., Davies, C., Sheldon, C. C., Jeffries, A. C. & Symons, R. H. (1988) *Nature (London)* **334**, 265–267.
6. Uhlenbeck, O. C. (1987) *Nature (London)* **328**, 596–600.
7. Haseloff, J. & Gerlach, W. L. (1988) *Nature (London)* **334**, 585–591.
8. Symons, R. H. (1991) *Crit. Rev. Plant Sci.* **10**, 189–234.
9. Forster, A. C. & Symons, R. H. (1987) *Cell* **50**, 9–16.
10. Ruffner, D. E., Stormo, G. D. & Uhlenbeck, O. C. (1990) *Biochemistry* **29**, 10695–10701.
11. Goodchild, J. & Kohli, V. (1991) *Arch. Biochem. Biophys.* **284**, 386–391.
12. McCall, M. J., Hendry, P. & Jennings, P. A. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5710–5714.
13. Tuschl, T. & Eckstein, F. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6991–6994.
14. Milligan, J. F., Groebe, D. R., Witherell, G. W. & Uhlenbeck, O. C. (1987) *Nucleic Acids Res.* **15**, 8783–8789.
15. Usman, N., Ogilvie, K. K., Jiang, M.-Y. & Cedergren, R. J. (1987) *J. Am. Chem. Soc.* **109**, 7845–7854.
16. Sampson, J. R. & Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1033–1037.
17. Fedor, M. J. & Uhlenbeck, O. C. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1668–1672.
18. Hofstee, B. H. J. (1952) *J. Biol. Chem.* **199**, 85–93.
19. Chamberlin, M. & Ring, J. (1973) *J. Biol. Chem.* **248**, 2235–2244.
20. Hertel, K. J., Herschlag, D. & Uhlenbeck, O. C. (1994) *Biochemistry* **33**, 3374–3385.
21. Dahm, S. C., Derrick, W. B. & Uhlenbeck, O. C. (1993) *Biochemistry* **32**, 13040–13045.
22. Dahm, S. C. & Uhlenbeck, O. C. (1991) *Biochemistry* **30**, 9464–9469.
23. Dichtl, B., Pan, T., DiRenzo, A. B. & Uhlenbeck, O. C. (1993) *Nucleic Acids Res.* **21**, 531–535.
24. Forster, A. C., Jeffries, A. C., Sheldon, C. C. & Symons, R. H. (1987) *Cold Spring Harbor Symp. Quant. Biol.* **52**, 249–259.
25. Sheldon, C. C. & Symons, R. H. (1989) *Nucleic Acids Res.* **17**, 5665–5676.