Interhelix Geometry of Stems I and II of a Self-Cleaving Hammerhead RNA†

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Received October 20, 1993®

ABSTRACT: In order to investigate the geometry of a self-cleaving hammerhead domain, an RNA heteroduplex has been constructed in which two of the three helix stems of the domain have each been elongated to 76 duplex base pairs (bp), resulting in an RNA molecule of *ca.* 160 bp. The heteroduplex molecule is capable of undergoing self-cleavage at neutral pH, upon addition of either Mg^{2+} or Mn^{2+}, but does not dissociate following cleavage. Using a combination of electrophoretic and hydrodynamic methods, as employed earlier to define the geometry of a four-way DNA branch [Copper & Hagerman (1989) *Proc. Natl. Acad. Sci. U.S.A. 86,* 7336–7340], we have determined that the elongated hammerhead stems are nearly collinear prior to self-cleavage. Following self-cleavage, and in the absence of Mg^{2+}, the angle between the two stems becomes much more acute and/or flexible; however, in the presence of Mg^{2+}, the cleaved structure appears to retain the geometry of the precleaved form (at least with respect to the interstem angle). It is also observed that the self-cleavage reaction is promoted by Mn^{2+} to a much greater extent than by Mg^{2+}, consistent with earlier observations. Finally, although the elongated helices appear to be nearly collinear in the uncleaved molecule, the electrophoretic mobility of that species is dramatically reduced with respect to linear control RNAs, indicating that caution should be exercised in the quantitative assignment of branch angles solely from gel retardation experiments.

Over the past several years, there has been a rapid growth of interest in reactions involving enzymatically active RNA species, examples of which include the M1 RNA from ribonuclease P (Guerrier-Takada et al., 1983, 1989; James et al., 1988; Forster & Altman, 1990), self-splicing group I introns (Zaug & Cech, 1986; Murphy & Cech, 1989; Green et al., 1990; Celander & Cech, 1991), self-splicing group II introns (Kwakman et al., 1990; Augustin et al., 1990; Mörl & Schmelzer, 1990), the hepatitis delta virus (Sharmeen et al., 1988; Perrotta & Been, 1990, 1991; Rosenstein & Been, 1990; Smith & Dinter-Gottlieb, 1991), the "hairpin" catalytic domain of the tobacco ringspot virus satellite RNA (Hampel et al., 1990), telomerase (Greider & Blackburn, 1989; Shippen-Lentz & Blackburn, 1990), and ribosomal RNA editing in mitochondria (Blum et al., 1990, 1991). One of the simplest of these systems is the self-cleaving RNA from certain plant pathogens (*e.g.*, the avocado sun blotch viroid; Uhlenbeck, 1987; Forster & Symons, 1987; Symons, 1989). These RNAs undergo site-specific cleavage as a means of processing large RNA precursors, generated by rolling-circle replication, into unit-length genomes. The small, phylogenetically conserved central catalytic domain (termed a "hammerhead" structure) has become the focus of special attention because of its potential use as an inhibitor of the translation of specific RNAs *in vivo* (Haseloff & Gerlach, 1988; Koizumi et al., 1989; Sarver et al., 1990). The hammerhead cleavage reaction takes place *via* an S_N2 mechanism (Koizumi & Ohtsuka, 1991), as reported earlier for the *Tetrahymena* self-splicing RNA (McSwiggen & Cech, 1989), and produces a cyclic 2′,3′-phosphate moiety at the 3′ side of the cleavage site (Hutchins et al., 1986; Symons, 1989). The cleavage reaction requires divalent cations, and a study of the binding of magnesium to a noncleavable hammerhead analogue yielded a magnesium binding constant of about 10^4 M^{-1} (Koizumi & Ohtsuka, 1991). Furthermore, Slim and Gait (1991) demonstrated that Mg^{2+} binds to the *pro-R* oxygen of the phosphate group at the cleavage site.

Despite a growing understanding of its catalytic mechanism, knowledge of the hammerhead structure remains elusive. A molecular dynamics study has suggested the presence of a pocket in the active site of the hammerhead that would be capable of accommodating a Mg^{2+} ion (Mei et al., 1989). NMR experiments thus far have been unable to determine the solution structure of the hammerhead, although such studies indicate that there is no substantial difference between the structure in the presence and absence of magnesium (Heus et al., 1990; Odai et al., 1990; Pease & Wemmer, 1990; Heus & Pardi, 1991). Furthermore, site-directed mutagenesis experiments (Fedor & Uhlenbeck, 1990; Ruffner et al., 1990; Koizumi & Ohtsuka, 1991; Slim & Gait, 1991), including those involving the use of thiophosphate (Ruffner & Uhlenbeck, 1990), substitution of ribonucleotides by deoxyribonucleotides (Perreault et al., 1990; Yang et al., 1990), or incorporation of modified nucleotides (Pieken et al., 1991), clearly demonstrated that the phylogenetically conserved bases in the catalytic domain, even those that may be far removed from the catalytic site itself, are all required for cleavage.

One of the goals of the current line of research is to define the long-range structure of the hammerhead (relative orientations of the helix stems), and the influence of both cleavage and base sequence on such structure. Our current approach is analogous to the approach used by Cooper and Hagerman, who determined the geometry of a DNA four-way junction using a combination of gel electrophoretic (Cooper & Hagerman, 1987) and hydrodynamic (Cooper & Hagerman, 1989) methods. We have estimated previously the rise per base pair and average flexibility of double-stranded RNA in

† This work was supported by grants from the Deutsche Forschungsgemeinschaft (F.-U.G.; Ga 351/1-2) and from the National Institutes of Health (P.J.H.; GM 35305).

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® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

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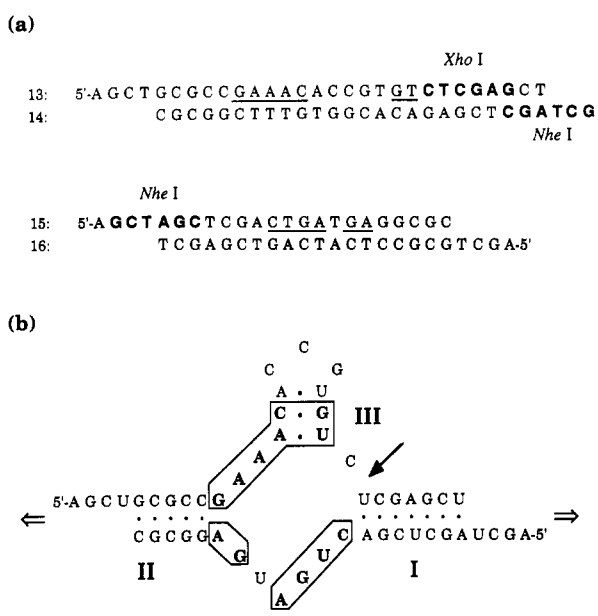

FIGURE 1: Outline of the construction of the hammerhead molecule, E[HH]. (a) Oligodeoxynucleotides used for cloning, with conserved hammerhead nucleotides on the nontemplate strand underlined and restriction sites indicated in boldface letters. The double-stranded oligonucleotides were inserted into the *Hin*dIII site of a pair of plasmids, which code a 136 bp random sequence in both orientations: pFU3A (A orientation) or pFU3B (B orientation) (Gast & Hagerman, 1991). The oligonucleotide sequences were chosen such that an *Nhe*I site is generated upon insertion into the *Hin*dIII site. Since a second site is present in the plasmid 44 bp downstream of the *Sma*I site, both the presence and the orientation of the insert could be determined by digestion of miniprep DNA with *Nhe*I, followed by analysis of the fragments on polyacrylamide gels. (b) Central domain of the elongated hammerhead, generated by transcription of the plasmids pFU3A-13 (oligomer 13, nontemplate strand) and pFU3B-15 (oligomer 15, nontemplate strand). Conserved sequences are boxed. The cleavage site is indicated by the arrow. Numbering of the arms is according to Symons (1989). Open arrows indicate stem extensions.

solution by electrophoretic and hydrodynamic (electrooptical) methods using molecules in the range of 136–226 base pairs (bp)[1] (Gast & Hagerman, 1991). In general, our hydrodynamic approach relies on the orientation of elongated molecules in an electric field. The rate of decay of birefringence, following removal of the field, can be used to determine either helical parameters (rise/bp, hydrodynamic radius) and flexibility (expressed as persistence length; Hagerman & Zimm, 1981; Hagerman, 1981) or the included angles of permanently curved molecules, if the helical parameters are already known (Hagerman, 1984, 1985a; Levene et al., 1986; Cooper & Hagerman, 1989).

In the current investigation, we have studied the self-cleaving hammerhead domain [using the core sequence studied by Uhlenbeck (1987)] by elongating two of the three stems of this RNA (stems I and II; Figure 1) with two 68 bp "reporter arms". This RNA molecule (termed an extended hammerhead, E[HH]) undergoes specific self-cleavage in the presence of magnesium or manganese ions at neutral pH. Our results demonstrate that, despite a dramatic reduction in the gel mobility of the extended hammerhead molecule (compared to linear duplex controls), the helix stems I and II (Figure 1) are nearly collinear. Moreover, its electrophoretic mobility in polyacrylamide and agarose gels is reduced further upon cleavage, but can be restored to nearly its original (precleaved) value in the presence of Mg^{2+}.

MATERIALS AND METHODS

General Methods. Many of the methods employed in the current work, including those for cloning, sequencing, plasmid isolation, transcription with T7 RNA polymerase, and purification of the annealed RNA products, are identical to those described in Gast and Hagerman (1991).

All template constructs employed the plasmids pFU3A and pFU3B (Gast & Hagerman, 1991), in which a specific DNA sequence had been cloned in both orientations downstream of a T7 promoter and upstream of an *Sma*I site. Runnoff transcription of these two plasmids (each cleaved with *Sma*I) yields transcripts which, when annealed, form a 136 bp double-stranded RNA (Gast & Hagerman, 1991). The extended hammerhead molecule, E[HH], was generated by ligating either of two double-stranded oligonucleotides (Figure 1a) into the central *Hin*dIII site of pFU3A and pFU3B; the oligonucleotides were left unphosphorylated in order to avoid multiple inserts. Plasmid minipreps were screened for the presence of inserts using *Eco*RV (since the original template includes a 122 bp *Eco*RV fragment; Gast & Hagerman, 1991), and for orientation of the inserts, using *Eco*RV–*Nhe*I double-digestion (Figure 1). Sequence-verified clones were numbered according to the orientation of the embedding sequence (A or B), and which strand of the oligonucleotides was transcribed (pFU3A-13 and pFU3B-15 were used for E[HH]). The linear standard RNA molecule (159 bp) was generated by transcription of the "bottom strand" of the hammerhead (from plasmid pFU3B-15), and by cloning and transcription of its exact complement in the "A" orientation plasmid (*i.e.*, pFU3A-16; Figure 1).

Cleavage Reactions and Their Analysis on Gels. Cleavage reactions involving magnesium or manganese ions were performed in Tris-HCl (50–100 mM, pH 7.9) with specified $MgCl_2$ or $MnCl_2$ concentrations (1–25 mM) and were analyzed for a range of temperatures from 4 to 42 °C (25° C unless specified otherwise). Cleavage of E[HH] with Pb^{2+} (0.5 mM) was performed at room temperature in 20 mM MOPS, pH 7, 15 mM $MgCl_2$, and 1.5 mM spermidine (Behlen et al., 1990). Aliquots of the self-cleavage reactions were withdrawn from the reaction mixture at specified times, and were quenched by adding an equal amount of gel loading buffer [0.25× TBE, 50 mM NaEDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, and 20% glycerol; 1× TBE: 90 mM Tris–borate (pH 8.3)/2.5 mM NaEDTA] followed by freezing at −20 °C. Both cleaved and uncleaved RNA species were analyzed on polyacrylamide gels ranging from 4% to 12% [ratio of acrylamide to bis(acrylamide), 29:1], and on 2.5% agarose gels in 0.5× TBE at room temperature. Alternative buffers and gel running temperatures were as indicated in the tables and figures. Electrophoresis buffers other than TBE were continuously recirculated. Gels were stained with ethidium bromide for 0.5–1 h, destained in distilled H_2O, and photographed on a long-wave UV transilluminator using a red filter. Kinetics were estimated by integrating the negatives of gel photographs using a laser scanner (LKB), followed by regression analysis of semilogarithmic plots.

The specificity of the self-cleavage reaction was determined by allowing the reaction to proceed to near-completion, denaturing the strands with glyoxal (Maniatis et al., 1982), and separating the fragments on an 8% sequencing gel. While one of the strands remained uncleaved, the other strand was

[1] Abbreviations: bp, base pair(s); DNA, deoxyribonucleic acid; ds, double-stranded; nt, nucleotide(s); EDTA, ethylenediaminetetraacetic acid; E[HH], elongated hammerhead RNA; *k*, unimolecular rate constant; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NaP_i, sodium phosphate buffer; RNA, ribonucleic acid; RT, room temperature; τ, decay time; TBE, Tris–borate buffer; TEB, transient electric birefringence; T_m, temperature of the midpoint of a thermal melting transition; $tRNA^{Phe}$, transfer ribonucleic acid (phenylalanyl-specific).

converted to two fragments of the expected sizes. Nuclease S1 cleavage experiments were performed as described in Gast and Hagerman (1991), followed by analysis on nondenaturating polyacrylamide gels.

Determination of Relative Electrophoretic Mobilities. Electrophoretic mobilities of RNA molecules were determined by comparing RNA band positions to DNA standards generated by *Hin*dIII cleavage of pGEM-7Zf(+) (Promega, Madison, WI), as well as to other RNA fragments, essentially as described in Gast and Hagerman (1991). In that paper, the relative mobility, μ_{rel}, was defined as the ratio of the distances traveled by the molecule of interest and a DNA standard of the same length. In the current paper, however, the mobility of an RNA molecule of interest (*e.g.*, E[HH]) is compared to the mobility of a linear double-stranded RNA standard of the same length. The latter was represented by a 159 bp dsRNA molecule.

Melting Experiments. Melting curves were determined in a Cary 219 spectrophotometer (Varian) at 260 nm using a 1-mL quartz cuvette with a 1-cm light path and equipped with an internal temperature probe. The temperature rise was approximately 0.5 °C/min. Since reannealing does not take place readily under low-salt conditions [as reported in Gast and Hagerman (1991)], only the temperature rise curves were recorded.

Transient Electric Birefringence Measurements. Transient electric birefringence (TEB) experiments (Fredericq & Houssier, 1973; Hagerman, 1985) were performed essentially as described in Gast and Hagerman (1991). The TEB instrument employs a 160-μL temperature-jacketed (±0.1 °C) cell with an electrode spacing of 2 mm. Pulse voltages ranged from 0.5 to 1.75 kV, with no detectable dependence of the decay times on field strength. Two buffers were used: TEB buffer A [5 mM NaP_i (pH 7.2)/0.125 mM EDTA] and TEB buffer B [10 mM Tris-HCl (pH 8.0)/0.05 mM EDTA]. RNA concentrations were *ca.* 0.1–0.3 mg/mL. "Self-cleaved" E[HH] was produced by incubation [50 mM Tris-HCl (pH 8.0)/20 mM $MgCl_2$] for 2 days, followed by 2-propanol precipitation in the presence of NaCl and excess EDTA, and desalting (*ca.* 80% cleaved). The integrity of the cleaved species was verified by gel analysis. Analysis of birefringence decay curves was performed on a macintosh IIci using a computer program written by one of the authors (F.-U.G.), or by using the program CONTIN (Provencher, 1982) as described earlier (Cooper & Hagerman, 1989). Most decay curves were single-exponential (except for a fast decay component of ≤20% of the total amplitude). The Macintosh (Microsoft QuickBasic) program can be obtained from F.-U.G. upon request. Decay times were corrected to 20 °C and the viscosity of water ($\tau_{20,w}$) as described elsewhere (Hagerman, 1985b; Gast & Hagerman, 1991). The principal (≥80% of the total amplitude) decay times of the elongated hammerhead molecules ($\tau_{E[HH]}$) were compared to the corresponding decay time of the linear 159 bp dsRNA molecule (τ_{159}). The ratios, R, of the decay times were then used to determine the included angle between the extended arms, as described by Cooper and Hagerman (1989).

RESULTS

Self-Cleavage and Gel Mobility of the Elongated Hammerhead Molecule. It is known that linear double-stranded RNA molecules migrate more slowly on polyacrylamide gels than do DNA fragments having the same number of base pairs (Bhattacharyya et al., 1990; Gast & Hagerman, 1991). The elongated hammerhead, E[HH], demonstrates a further reduction in electrophoretic mobility on every gel system investigated, including 2.5% agarose (Figure 2, Table 1). On 8% polyacrylamide gels, the 162 bp long (mean length of both strands) E[HH] has the same electrophoretic mobility as a 310 bp DNA molecule, whereas the linear, 159 bp standard migrates at a rate equivalent to a DNA molecule of 210 bp (Figure 2a). This behavior may reflect the presence of the short stem III, which is expected to impede the migration of E[HH] through the gel [e.g., see Cooper and Hagerman (1987, 1989)]. This suggestion is reinforced by the observation (Table 1) that for both cleaved and uncleaved E[HH] the relative mobilities approach unity, indicating that the E[HH] molecules do not run appreciably more slowly than their linear counterpart under conditions of free electrophoresis.

The mobility of the already retarded E[HH] molecule is reduced still further upon cleavage, running as a 440 bp DNA molecule on 8% gels (Figure 2a). This latter behavior is similar to the gel behavior of double-stranded DNA molecules possessing short (1–5 nt) single-stranded gaps (Mills et al., 1994), and suggests that not only stable curvature [for reviews, see Hagerman (1990, 1992); Crothers et al., 1990] and decreased net charge (Gast & Hagerman, 1991) but also points of increased flexibility can lead to reduced mobilities. Limited nicking of the single-stranded regions of E[HH] with the single-strand-specific nuclease S1 also results in cleavage intermediates with mobilities similar to the self-cleaved molecule (not shown); however, more complete cleavage with S1 leads to two half-molecules, as expected.

Kinetics of the Self-Cleavage of the Elongated Hammerhead. The rates of E[HH] self-cleavage appear to be somewhat slower (Table 2) than rates observed previously for other hammerhead species (Ruffner et al., 1989; Uhlenbeck, 1987; Fedor & Uhlenbeck, 1990; Yang et al., 1990; Slim & Gait, 1991; Koizumi & Ohtsuka, 1991; Pieken et al., 1991). The current observations are consistent with other results (Forster et al., 1988; Sheldon & Symons, 1989; Davies et al., 1991; Epstein & Pabòn-Peña, 1991) which suggest that an intact, stable helix III is needed for efficient cleavage. This proposal is strengthened by the observation (Amiri and Hagerman, unpublished results) that increasing stem III to nine base pairs reduces the self-cleavage half-times to well under 1 min, without any apparent alteration of the geometry of stems I and II.

We have observed that self-cleavage is promoted to a greater degree by Mn^{2+} than by Mg^{2+}, since the cleavage of E[HH] in the presence of Mn^{2+} is about 1 order of magnitude faster than in the presence of Mg^{2+}, at least for lower concentrations of these cations. These results are in agreement with results obtained with short hammerheads (Uhlenbeck, 1987; Slim & Gait, 1991; Pieken et al., 1991; Fedor & Uhlenbeck, 1992). However, there is a sharp drop in the Mn^{2+}-catalyzed cleavage rate above pH 8, due to the formation of $MnCl_2$ precipitates in alkaline buffers. The observed drop in rate above pH 8 also indicates that the metal-induced cleavage of E[HH] is not an artifact of general base hydrolysis of the single-stranded RNA regions, but represents metal-ion-dependent self-cleavage. We observe no corresponding drop in rate with Mg^{2+}. Cations such as spermidine neither induce nor accelerate the cleavage reaction under these conditions (data not shown), in accord with earlier results (Uhlenbeck, 1987); however, Dahm and Uhlenbeck (1991) observed that spermine can replace a "structural" Mg^{2+}, thus reducing the concentration of Mg^{2+} necessary for efficient cleavage.

The cleavage reaction appears to be biphasic (Table 2), even though E[HH] is purified as a single band from polyacrylamide gels. This latter behavior is being investigated

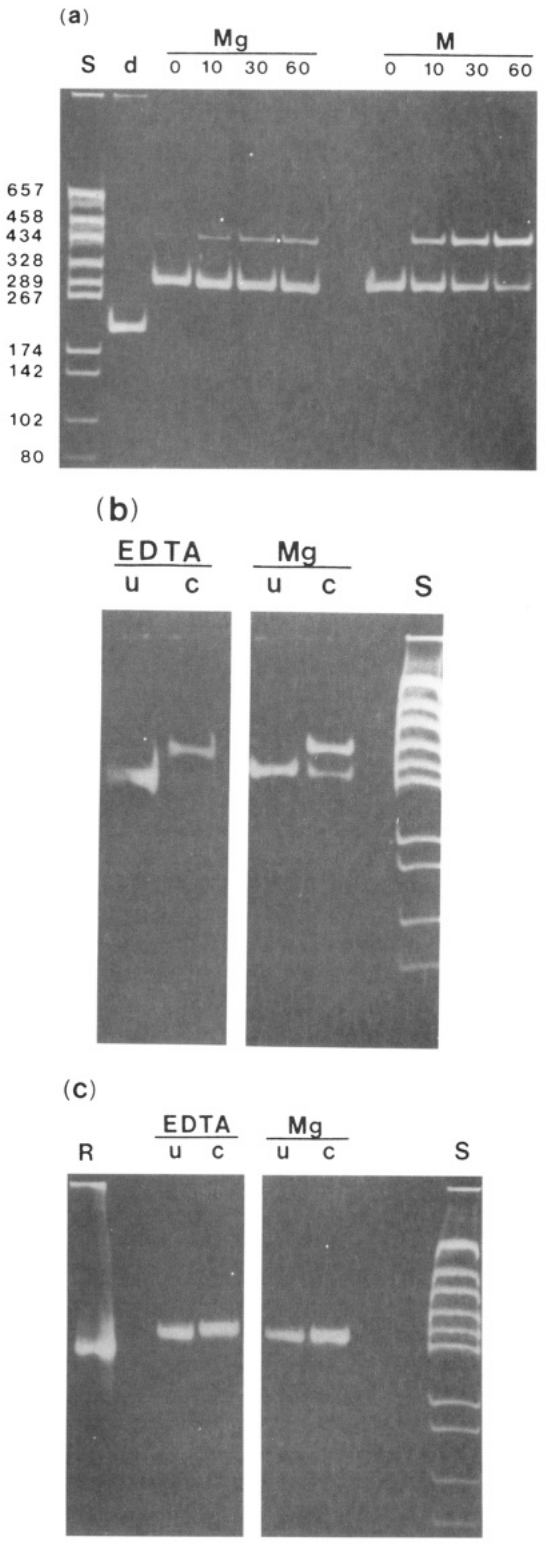

FIGURE 2: Gel analysis of the products of self-cleavage of E[HH]. (a) Time course of cleavage. E[HH] (0.06 mg/mL) was incubated in 30 μL of a mixture of 100 mM Tris-HCl, pH 7.6, and 20 mM divalent metal chloride at 37 °C; 4-μL aliquots were withdrawn, stopped with 6 μL of loading buffer (see Materials and Methods), and analyzed on an 8%, 0.5× TBE gel at room temperature. Time points: 0, 10, 30, and 60 min. Left side of the gel, incubation with $MgCl_2$; right side, incubation with $MnCl_2$. S, pGEM-72f(+)/*Hae*III (DNA) standard; d, 159 bp dsRNA standard. (b) Electrophoretic mobilities of uncleaved (u) and cleaved (c) E[HH] at 4 °C in the presence of 10 mM Tris-HCl (pH 8)/50 μM EDTA added to the gel buffer. The E[HH] species were preincubated for 15 min at room temperature, either in 2 mM EDTA (EDTA) or in 2 mM $MgCl_2$ (Mg). (c) Electrophoresis as in (b), except that the EDTA in the gel buffer has been replaced by 10 μM $MgCl_2$. The 180 bp dsRNA standard (unpurified following the transcription) is at the left.

Table 1: Compilation of the Relative Mobilites of E[HH] on Agarose and Polyacrylamide Gels[a]

% gel[c]	additions	μ_{rel}[b] E[HH]	μ_{rel}[b] E[HH] cleaved	ratio[d]
4		0.93	0.84	0.90
6		0.87 ± 0.01	0.73 ± 0.2	0.84
8		0.81 ± 0.02	0.60 ± 0.1	0.74
10		0.78 ± 0.02	0.57 ± 0.03	0.73
12		0.77	0.52	0.68
2.5 (agarose)		0.96	0.91	0.95
8	100 mM NaCl	0.72	0.63	0.88
8	3 mM $MgCl_2$	0.86	0.90	1.05
8	60 °C	0.84	0.67	0.80
8	0.1 mM $MgCl_2$ (–EDTA), 55 °C[e]	0.87	0.75	0.86
8	5 mM $MgCl_2$, 60 °C	0.82	0.78	0.95
8	Tris[f] + 50 μM EDTA, 4 °C	0.74	0.58	0.78[g]
8	Tris[f] + 10 μM $MgCl_2$, 4 °C	0.82	0.82	1.00[g]
8	Tris[f] + 50 μM $MgCl_2$, 4 °C	0.85	0.87	1.02[g]
8	Tris[f] + 20 μM $MgCl_2$, RT	0.81	0.75	0.93[g]

[a] If not otherwise stated, electrophoresis conditions were 0.5× TBE [49 mM Tris–borate (pH 8.3)/1.25 mM Na_2EDTA], RT (=24 ± 1 °C), and ~10 V/cm gel, except for 100 mM NaCl, where it was 5 V/cm (13-cm-long gels). [b] Relative mobilities, μ_{rel} (±1 standard deviation, if more than two gels were run), were determined as described under Materials and Methods, using a linear standard molecule of 159 bp. Linear regression of μ_{rel} values for polyacrylamide gel concentrations over the range 4–10% leads to μ_{rel} (0% gel) values of 1.03 (E[HH], cleaved) and 1.01 (E[HH], uncleaved). [c] Percentage acrylamide gel, unless noted otherwise. [d] Ratio of μ_{rel} for E[HH] cleaved and E[HH] uncleaved. [e] EDTA was left out of the 0.5× TBE buffer in this case. [f] Conditions for these gel systems were 10 mM Tris-HCl, pH 8 (instead of 0.5× TBE), with buffer recirculation. [g] μ_{rel} data of samples preincubated with ~2 mM EDTA or $MgCl_2$ are averaged, due to their comparable values.

Table 2: pH Dependence of Self-Cleavage of the Elongated Hammerhead Molecule, E[HH][a]

pH	fraction[b]	k (min^{-1})	pH	fraction[b]	k (min^{-1})
		Fast Phase			
7	0.33	0.0047	8.5	0.49	0.038
8	0.45	0.020	9	0.50	0.11
		Slow Phase			
7	0.67	$<10^{-4}$	8.5	0.51	0.002
8	0.55	0.006	9	0.50	0.007

[a] Cleavage buffer was 100 mM Tris-HCl (pH as indicated)/25 mM $MgCl_2$; temperature, 25 °C. [b] Decay curves displayed apparent biphasic kinetics of cleavage; the fractional amplitude associated with each phase is given in column 2.

further (Amiri and Hagerman, unpublished results), and may reflect the existence of more than one conformer within the core (conserved) region of the hammerhead. Complex (multiphasic) self-cleavage reactions have also been observed with hepatitis delta virus (Thill et al., 1993), although the authors did not incorporate the multiphasic character of the cleavage reactions into their analysis. Multiphasic behavior has not been reported for cleavage reactions involving small RNA molecules, possibly indicating that strand dissociation is more rapid than the slower cleavage rates. The rates of self-cleavage observed with E[HH] do not vary strongly between 25 and 42 °C, suggesting that the enthalpies of activation for self-cleavage are small in that temperature range. Previously published values for the activation enthalpy (Uhlenbeck, 1987) must be interpreted with caution (O. Uhlenbeck, personal communication), since those values were not corrected for the enthalpy of helix formation. In this regard, one advantage of the current system is that the purification of E[HH] prior to cleavage eliminates contributions due to the enthalpy of hammerhead formation. Moreover, under the conditions used for the birefringence and gel studies, cleaved E[HH] cannot dissociate, since the

T_m of 0.02 μM E[HH] (2 μg/mL) is 77 °C in TEB buffer A, 89 °C in the presence of 50 mM NaCl, and 92 °C in the presence of 100 mM NaCl, and is not measurable (>95 °C) in the presence of 1 mM $MgCl_2$ (TEB buffer A).

Mg^{2+}-catalyzed self-cleavage, observed at 4 °C over an 8-day period, or at 25 °C over a 6-day period, could not produce more than 95–98% of the cleaved species. We have observed, with an E[HH] derivative possessing a 9 bp helix III, that the cleavage reaction is reversible, producing *ca.* 3% religated material (Amiri and Hagerman, unpublished results). This latter observation is consistent with the generation of a cyclic 2′,3′-phosphate during the self-cleavage reaction (Symons, 1989). Since the energy of the phosphodiester bond is preserved in the cyclic intermediate, it would be possible for the backward reaction to take place (with a resulting equilibrium at about 97% cleavage). The same phenomenon was reported earlier by Tsagris et al. (1991), who observed that when ribonuclease T1 was used to trim a viroid precursor molecule, cleavage of the precursor with T1 produced significant amounts (*ca.* 2–5%) of the fully ligated circular viroid product. The explanation provided by those authors was that the cyclic 2′,3′-phosphate, which is formed as an intermediate, could be used by the nuclease for the ligation reaction under conditions where the RNA structure facilitated the close approximation of the ligated ends.

Finally, we have observed that Pb^{2+} ions cleave E[HH] (data not shown), as has been demonstrated previously for unmodified $tRNA^{Phe}$ transcripts (Behlen et al., 1990) and for 5S RNA (Ciesiolka et al., 1992). In contrast to Mg^{2+} (or Mn^{2+}), which promotes cleavage next to a specific residue (Figure 1), Pb^{2+}-induced cleavage is relatively nonspecific, producing two half-molecules. Ca^{2+}, on the other hand, does not cleave E[HH] (data not shown). These observations parallel results obtained with RNA model compounds, where such molecules were hydrolyzed efficiently with Eu^{3+}, Tb^{3+}, and Y^{3+}, less efficiently with Zn^{2+}, Pb^{2+}, and Mg^{2+}, and not at all with Ca^{2+} (Breslow & Huang, 1991).

Behavior of the Elongated Hammerhead RNA Molecule on Gels Containing Magnesium. The difference in electrophoretic mobility between E[HH] and its cleaved product is not substantially altered between two different buffer systems (TEB buffer B and 0.5× TBE; Table 1), even though those buffers differ significantly in buffer ion concentration. These results are in line with previous observations pertaining to the electrophoretic behavior of dsRNA (Gast & Hagerman, 1991). However, for either buffer system, the addition of Mg^{2+} results in a shift in the mobility of the cleaved species to a value that is equal to or slightly greater than that of the uncleaved form (Table 1; Figure 2b), suggesting that Mg^{2+} ions play both structural and catalytic roles (the mobility of the uncleaved form is not significantly influenced by Mg^{2+}). This effect is observed at Mg^{2+} concentrations as low as 10 μM at 4 °C. Moreover, spermidine, although itself unable to promote cleavage, produces a similar shift in the mobility of the cleaved species. These observations are in accord with NMR studies that have shown that the structure of the uncleaved hammerhead molecule is not substantially different in the presence and absence of Mg^{2+} (Heus et al., 1990; Odai et al., 1990; Pease & Wemmer, 1990; Heus & Pardi, 1991). Addition of low amounts of EDTA (buffer lacking Mg^{2+}) restores the original mobility of the cleaved molecule (Table 1, Figure 2b). Furthermore, preincubation with high amounts of either EDTA or $MgCl_2$ does not drastically alter the migration of cleaved E[HH] on gels containing 10 μM Mg^{2+} (Figure 2c), indicating that the Mg^{2+} concentration (10 μM) in that gel is probably greater than the value of the dissociation constant.

Table 3: Birefringence Decay Times of the Linear 159 bp Standard and the Elongated Hammerhead Molecule, E[HH][a]

	$\tau_{20,w}$ (μs)		
		E[HH]	
buffer[b]	159 bp molecule	uncleaved	cleaved
buffer A + EDTA[c]	1.61 ± 0.05	1.41 ± 0.08	≈0.4[d]
buffer B + EDTA[c]	1.65 ± 0.08	1.52 ± 0.05	≈0.6[d]
buffer A + Mg^{2+} [e]	1.53 ± 0.06	1.58 ● 0.03	1.40 ± 0.13
buffer B + Mg^{2+} [e]	1.53 ± 0.06	1.47 ● 0.08	1.48 ± 0.05

[a] Decay times were measured at 4.0 ± 0.2 °C, and were corrected to 20 °C. Each decay time is averaged from at least six experiments at different voltages. Each experiment comprises an average of 32–64 individual measurements. Results are reported ±1 standard error. [b] Buffer A: 5 mM NaP_i (pH 7.2)/0.125 mM EDTA. Buffer B: 10 mM Tris-HCl (pH 8)/0.05 mM EDTA. [c] Results of experiments in the presence of EDTA are essentially averages from experiments in the original TEB buffer, which contains small amounts of EDTA, and from experiments in the presence of larger amounts of EDTA (up to *ca.* 2 mM). However, since the small amounts of EDTA present in the TEB buffers were insufficient to convert the cleaved hammerhead to the "EDTA" form, only the results of those experiments with higher EDTA concentrations (*ca.* 1–6 mM) were averaged for the cleaved species. [d] τ values for the cleaved E[HH] molecule are only approximate, due to the complex nature of the decay curves and to possible coupling to the instrument response time. [e] Mg^{2+} experiments were averaged for the range 1–2 mM $MgCl_2$, since no significant difference could be found between the decay times at 1 and 2 mM $MgCl_2$.

Table 4: Ratios of the Birefringence Decay Times of Extended Hammerhead Molecules, E[HH], to the Linear 159 bp Molecule

condition[a]	R^b	condition[a]	R^b
uncleaved, +EDTA	0.90	cleaved, +Mg^{2+}	0.95
uncleaved, +Mg^{2+}	1.00	cleaved, +EDTA	≈0.3

[a] Results obtained in buffer A and buffer B were averaged; EDTA or Mg^{2+} conditions are described in Table 3. [b] $R = \tau_{20,w}(E[HH])/\tau_{20,w}$(159 bp control).

Therefore, the binding constant of Mg^{2+} to the cleaved hammerhead would appear to be at least 10^5 M^{-1} at 4 °C under conditions used for the birefringence experiments. The presence of high amounts of NaCl also narrows the mobility gap between the cleaved and the uncleaved hammerhead molecule. However, in this instance, it is the mobility of the uncleaved E[HH] that is altered (Table 1). The origin of this effect of NaCl is unknown; however, NaCl neither accelerates nor inhibits the hammerhead cleavage reaction (Uhlenbeck, 1987; Gast and Hagerman, unpublished observations).

Transient Electric Birefringence Measurements. In the presence of magnesium, the birefringence decay times of uncleaved E[HH] are identical to those obtained under the same conditions for the linear control molecule (159 bp; Tables 3 and 4; Figures 3 and 4). These results suggest that, within our current limits of experimental uncertainty, helix stems I and II are collinear. Moreover, the close agreement between the two decay times suggests that the central, conserved region is relatively extended along the axis of stems I and II, since the 159 bp control contains pure double helix between those stems (Figure 4). It should be noted that the birefringence measurements can be performed in a time range during which cleavage is only barely detectable; thus, measurements performed in the presence of magnesium reflect the structure prior to cleavage. It should also be noted that the decay time of E[HH] is not influenced by the presence of stem III, since an E[HH] derivative with a 9 bp stem III yields an identical decay time (Amiri and Hagerman, unpublished results). This latter observation is not surprising, since rotational diffusion rates of elongated molecules are quite insensitive to points of drag near the center of hydrodynamic resistance. In the absence of magnesium, the decay time for E[HH] is essentially

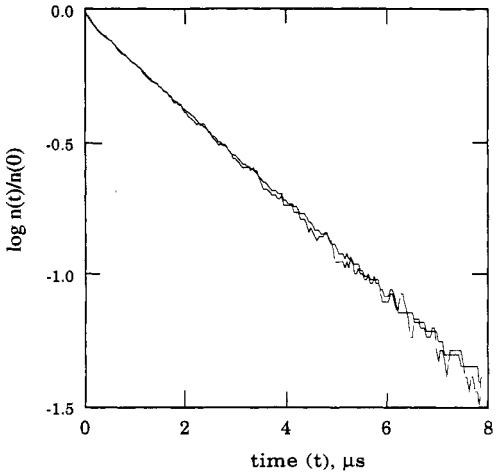

FIGURE 3: Birefringence decay curves for the elongated hammerhead, E[HH], and the 159 bp dsRNA control, in birefringence buffer A, pH 7.2; temperature, 4 °C. The individual curves represent averages of four experiments (see Table 3).

unchanged while that of the 159 bp control is slightly larger (Table 3). These two observations presumably reflect both the stiffening of the helices themselves (larger τ values for the 159 bp molecule) and a general loosening of the conserved region (R reduced to 0.9; Table 4).

Self-cleavage of E[HH] yields a species whose behavior in the absence of magnesium is that of a substantially bent or flexibly-hinged molecule. The dramatic reduction in the mobility of cleaved E[HH] relative to its uncleaved counterpart provides strong qualitative evidence for a substantial degree of bending and/or flexibility at the site of cleavage (Table 1). This conclusion is reinforced by the observation of a dramatic drop in the birefringence decay time of the self-cleaved species (Table 3). Due to the presence of a dominant fast component (70–80%) in the birefringence decay curve of the cleaved E[HH] species (no Mg^{2+}), as well as a substantial reduction in the decay time of the slow phase, we have not attempted to obtain a precise estimate for the terminal decay time of the cleaved species in the absence of Mg^{2+}; however, it is considerably less than 1 μs (Table 3).

In the presence of millimolar concentrations of Mg^{2+}, both the birefringence decay and the gel electrophoretic behavior of the cleaved E[HH] molecule are nearly identical to those of its uncleaved counterpart (Tables 1, 3, and 4). This characteristic of the cleaved molecule in magnesium stands in sharp contrast to its behavior in the absence of magnesium, and suggests that magnesium stabilizes a hammerhead conformation similar to one involved in the process of self-cleavage. One potential consequence of the postcleavage stabilization by magnesium is that the ends generated by the cleavage reaction (5′-hydroxyl and cyclic 2′,3′-phosphate) may remain favorably positioned to carry out the reverse reaction.

DISCUSSION

We have produced a self-cleaving hammerhead RNA molecule whose double-helical stems I and II have been elongated substantially, yielding a molecule (extended hammerhead; E[HH]) that is suitable for hydrodynamic studies and that is incapable of strand exchange and multiple turnover. The rate of self-cleavage of the extended hammerhead is 1–2 orders of magnitude slower than rates reported for other, shorter hammerhead molecules (Ruffner et al., 1989; Uhlenbeck, 1987; Fedor & Uhlenbeck, 1990; Yang et al., 1990; Slim & Gait, 1991; Koizumi & Ohtsuka, 1991; Pieken et al., 1991). In particular, the observed rate of self-cleavage of

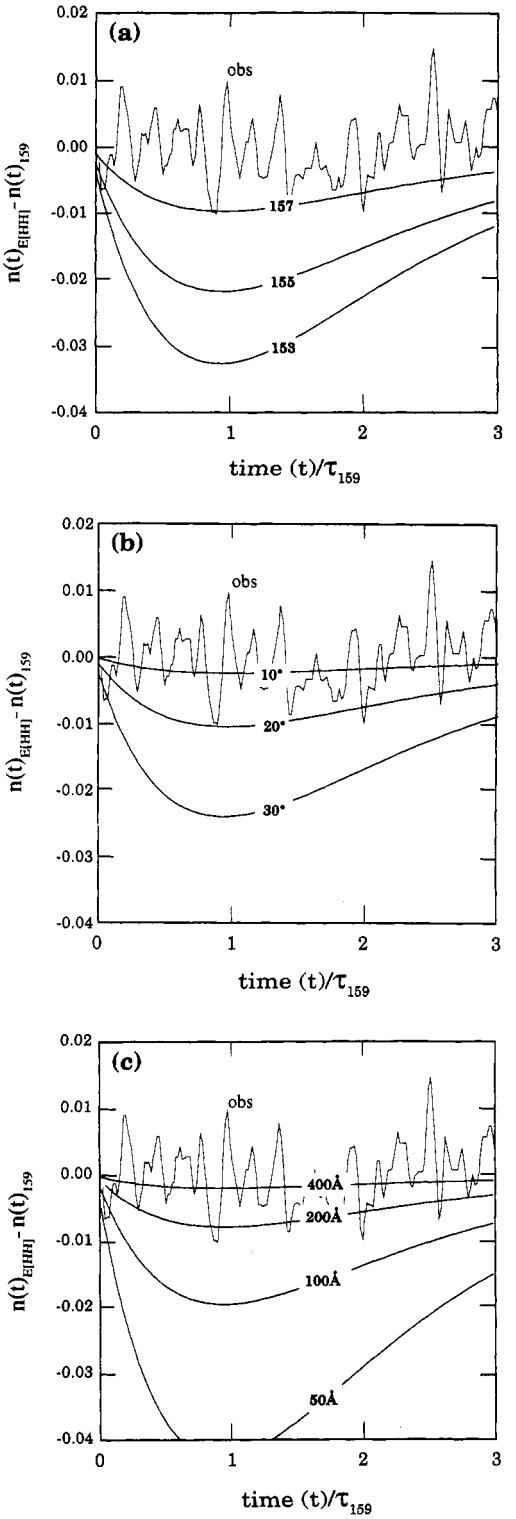

FIGURE 4: Plots of the differential birefringence decay of the two curves displayed in Figure 3. In each of the three panels, "obs" represents the observed differential decay curve, $n(t)_{E[HH]} - n(t)_{159}$. The computed curves in each panel reflect the expected difference curves, following variation of a single parameter, and are generated using a modified version of the program DIFFROT used previously to determine the rotation diffusion constants of wormlike chains containing positions of curvature (Cooper & Hagerman, 1989). All curves are plotted as a function of the dimensionless parameter t/τ_{159}. RNA helix parameters include a persistence length of 600 Å, a helix rise of 2.7 Å, and a hydrodynamic radius of 13 Å (Gast & Hagerman, 1991). (a) Difference curves expected for an RNA molecule foreshortened by 2 (157), 4 (155), or 6 bp (153). (b) Difference curves expected for a 159 bp RNA molecule possessing a bend of 10°, 20°, or 30° at its center. (c) Difference curves expected for a 159 bp RNA molecule possessing a central (4 bp) region of increased flexibility, with reduced persistence lengths as indicated.

E[HH] appears to be slower than the corresponding value for the hammerhead sequence upon which the core of E[HH] is based (Ruffner et al., 1989). However, any difference in rates is probably localized to the region around helix III, since elongation of that helix stem to 9 bp results in cleavage half-times below 1 min, without any significant change in the relative orientations of helices I and II (Amiri and Hagerman, unpublished results). One possible explanation for the difference in rates is that the reaction requires a stable stem III. Koizumi and Ohtsuka (1991) recently demonstrated that a short hairpin loop in place of helix III leads to very slow cleavage, compared to the rate of cleavage observed with an intact, apparently less restrained helix III. Furthermore, several groups have found that the species most likely to be active in the cleavage reaction is the double-hammerhead, which can form with any hammerhead sequence (Forster et al., 1988; Sheldon & Symons, 1989; Davies et al., 1991; Epstein & Pabòn-Peña, 1991). Formation of the double-hammerhead may be critical for ribozymes in which helix III is very short, since the double-hammerhead allows for an elongated, well-formed third helix. Few controls have been done thus far to exclude a double-hammerhead in self-cleavage experiments. Ruffner et al. (1989) employed mutant hammerhead species to assess the role played by double-hammerhead structures in self-cleavage. Those authors did observe cleavage via a double-hammerhead intermediate; however, they were unable to determine structure-specific rates of cleavage for the double-hammerheads. E[HH], on the other hand, provides no opportunity for either strand exchange or formation of the double-hammerhead molecule. This feature of E[HH], coupled with its slow cleavage rate, thus provides further support, albeit indirect, for a double-hammerhead mode of cleavage for molecules possessing a short stem III. In this regard, it should be noted that during the process of annealing the two transcripts comprising E[HH], additional bands are produced which demonstrate much lower mobilities than E[HH] on polyacrylamide gels. These bands, removed during purification of E[HH], and representing only a small fraction of the total annealed product, disappear in the presence of $MgCl_2$ or $MnCl_2$ within a few minutes, presumably through self-cleavage events. We have not pursued the characterization of these latter species; however, these bands are not observed with elongated hammerheads containing 9 bp in stem III.

The hammerhead RNA elongated at helices I and II is retarded on polyacrylamide gels compared to a linear (159 bp) control molecule. Rotational diffusion experiments, however, clearly indicate that the two extended helices are essentially collinear (Figures 3 and 4). Analysis of the birefringence experiments using the Monte-Carlo method as employed by Cooper and Hagerman (1989) indicates that the deviation for collinearity cannot be more than 10–15° (Figure 4). In their transient electric dichroism study of intrinsically curved DNA, Diekmann and Pörschke (1987) suggested that curved molecules might be straightened by the electric field, failing to return to their previous conformations within the time scale of the experiment. However, their measured decay times showed a remarkable dependence on the applied electric field. In contrast, none of the RNA molecules employed in the current study has shown any dependence of the terminal decay time on the strength of the applied field, consistent with earlier observations with branched DNA molecules (Cooper & Hagerman, 1989). While this latter observation does not completely rule out a straightening of the molecule in the field, it does indicate that conformational equilibration following reversal of the field is rapid compared to the time scale of the birefringence decay measurements. This position is given further support by the observation that in the presence of excess EDTA, the cleaved E[HH] product does show a dominant fast decay component, which indicates that the cleaved molecule is able to return rapidly to its prefield conformation.

Self-cleavage of E[HH] generates a molecule whose electrophoretic mobility in the absence of magnesium is further reduced from the already low mobility of the uncleaved molecule. This observation is paralleled by a dramatic reduction in the birefringence decay time of the cleaved species following treatment with EDTA. Taken together, these observations could reflect either a fixed, acute bend between stems I and II (Figure 1) or the creation of a point of substantial flexibility within the core of the cleaved hammerhead. While the current study does not completely resolve this issue, the following observations suggest that in the absence of magnesium the cleaved core exhibits substantial flexibility: (i) Birefringence decay experiments in which cleaved E[HH] has been treated with EDTA reveal a dramatic increase in the fast component of the decay curve, suggesting segmental reorientations; (ii) the extrapolated relative mobility, μ_{rel} (0% gel), of the cleaved species is not significantly different from that of the uncleaved species, arguing against an acute, fixed-angle bend; (iii) studies of DNA molecules with central points of increased flexibility indicate that hingelike flexibility can also result in substantial gel retardation (Mills et al., 1994). The apparent increase in the flexibility of the cleaved product in the absence of magnesium suggests that at least one role of the divalent cation is that of stabilizing a particular structure within the hammerhead core, a function that can at least partially be subserved by spermidine (see below).

The gel results presented in Table 1 indicate that magnesium has a strong binding constant to the cleaved E[HH] species at 4 °C (at least 10^5 M^{-1}). Moreover, the binding constant appears to decrease with increasing temperature, although this last effect needs further characterization. In view of the temperature dependence of the magnesium effect in gels, the estimated binding constant for Mg^{2+} accords well with circular dichroism experiments performed with noncleavable hammerhead molecules (Koizumi & Ohtsuka, 1991), which yielded a binding constant of 10^4 M^{-1} for Mg^{2+} at 18 °C. The binding constant would appear to reflect a large disparity between the concentration required to fully stabilize the cleaved hammerhead ($\leq$0.01 mM; Table 1) and the concentration necessary to achieve the maximal rate of cleavage (>20–50 mM). The current study does not resolve this difference; however, one can speculate about possible reasons for the difference. First, the binding constant of magnesium to the original (precleaved) hammerhead molecule, which cannot be determined from our experiments, may be different from the binding constant of Mg^{2+} to the cleaved molecule. Second, the kinetics of the reaction might involve the binding of more than one molecule of magnesium, with the first equivalent being relegated to a structural role, while the second equivalent participates in the cleavage reaction itself. Koizumi and Ohtsuka (1991) have determined that 1.2–1.9 magnesium ions bind to the hammerhead, a result that is not inconsistent with the two-cation model. Moreover, Dahm and Uhlenbeck (1991) have observed that 2–3 Mg^{2+} ions appear to act cooperatively in the hammerhead cleavage reaction. In their experiments, one of the magnesium ions could be replaced by spermine.

A major uncertainty at present is the nature of the structure within the hammerhead core. Turner and co-workers (SantaLucia et al., 1990) have shown that mismatches of the form AG·AG and GA·GA (both strands written in the 5′-to-3′

orientation) are significantly more stable than other mismatches. While the first set of dinucleotides apparently form base pairs, the second ones do not; thus, other factors must contribute to the increased stability of the second pairing. The second mismatch corresponds to the sequence found in the hammerhead molecule (Figure 1), where the GAAA sequence of the "top" strand is balanced by the GA sequence of the "bottom" strand. Extensive base stacking in the absence of base pair hydrogen bond formation has been predicted for this region in a molecular dynamics study of the hammerhead (Mei et al., 1989). Moreover, there is increased evidence for unusual nucleotide interactions in RNA, including the *syn* conformation of nucleotides and extensive stacking (*e.g.*, a G1·A4 base pair and a hydrogen bond between base A3 and a phosphate in the GNAA tetraloop; Heus & Pardi, 1991), as well as a hydrogen bond between base C3 and a phosphate in the UNCG tetraloop (Cheong et al., 1990). Such interactions may stabilize the hammerhead molecule while facilitating cleavage.

Our own observation (Figure 4a) that the hammerhead core is extended along the stem I–stem II axis further serves to limit the possible combinations of base–base interactions between the upper and lower strands. As can be shown with model substrates, slow, metal-induced RNA cleavage can be induced at any RNA phosphodiester bond in solution (Breslow & Huang, 1991). Hence, the main task a catalytic (or self-cleaving) RNA has to achieve is to make a particular bond susceptible for cleavage through optimization of the interaction with the metal catalyst while protecting all other bonds. The approach utilized in the current study should allow one to examine directly the structural consequences of base substitutions within the conserved region of the hammerhead, without the requirement of catalytic activity.

ACKNOWLEDGMENT

We thank J. B. Mills for help with the oligonucleotide syntheses and Dr. R. G. Bellomy, Dr. J. P. Cooper and M. Friederich for their scientific input. We are also indebted to Dr. H. L. Sänger for discussions regarding a possible backward reaction, and to Dr. O. C. Uhlenbeck and his group for their kind gift of T7 RNA polymerase, and for useful discussions. F.-U.G. also acknowledges the support of the Max-Planck-Gesellschaft during the preparation of this paper.

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