Reaction Conditions and Kinetics of Self-Cleavage of a Ribozyme Derived from *Neurospora* VS RNA†

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Received August 21, 1992; Revised Manuscript Received December 7, 1992

ABSTRACT: We have investigated the self-cleavage reaction performed by a ribozyme that contains 164 nucleotides of *Neurospora* VS RNA. Self-cleavage requires a divalent cation, magnesium being more effective than manganese or calcium. Spermidine or monovalent cations stimulate the reaction but cannot replace magnesium. The temperature optimum is rather broad, around 40–50 °C. Unlike some other ribozymes, VS self-cleavage is inhibited by even low concentrations of urea or formamide. The rate of cleavage is the same from pH 5.5 to 8.9, suggesting either that hydroxide is not directly involved in the cleavage chemistry or that a step that precedes the actual cleavage event is rate-limiting.

A small number of RNAs, called ribozymes, are known to catalyze cleavage reactions using either themselves or other RNAs as substrates [reviewed by Symons (1992)]. A given ribozyme cleaves the target phosphodiester bond in a characteristic fashion: products or intermediates with 3′-hydroxyl and 5′-phosphate termini are formed by group I (Cech, 1987) and group II (Michel et al., 1989) introns and RNase P (Guerrier-Takada et al., 1983); 2′,3′-cyclic phosphate and 5′-hydroxyl termini are produced by the genomic and antigenomic strands of hepatitis delta virus (HDV; Sharmeen et al., 1988; Kuo et al., 1988), the minus strand of the satellite of tobacco ringspot virus [(–)STRV; Buzayan et al., 1986], a *Neurospora* mitochondrial plamid transcript (VS RNA; Saville & Collins, 1990), and several RNAs that contain a common structural motif called the hammerhead [Forster & Symons, 1987a; reviewed by Symons (1992)].

Among the ribozymes that produce cyclic phosphate termini, there appear to be different structural types: self-cleavage of HDV and VS RNAs requires only a single nucleotide upstream of the cleavage site (Perrotta & Been, 1990; Guo et al., 1993). Hammerhead RNAs require at least three upstream nucleotides, two of which are presumably required to base pair with distant nucleotides to form a helix called stem III [reviewed by Symons (1992)] as well as possibly for other interactions. An upstream helix is also required for trans cleavage by (–)STRV (Hampel et al., 1990), although this helix may not be required for self-cleavage (van Tol et al., 1991). Each of these ribozymes has a distinctive secondary structure, certain features of which are known to be important for cleavage. It is not obvious from the secondary structures how the cleavage site is specified, and it remains to be determined if any similarities in higher order structure will emerge that might reveal common features among the ribozymes.

Current structural models also do not provide a thorough explanation of the functional properties of the ribozymes. For example, in addition to self-cleavage, (–)STRV and VS RNAs ligate much more efficiently than other ribozymes (Buzayan et al., 1986; Saville & Collins, 1991). Also, ribozymes derived from HDV are distinctive in being active at high temperatures and in the presence of high concentrations of denaturants (Rosenstein & Been, 1990; Wu & Lai, 1990; Smith & Gottleib, 1991). Here we report our investigation of the functional properties of a VS ribozyme, including the effects on self-cleavage of several factors that would be expected to affect RNA structure and function.

MATERIALS AND METHODS

Synthesis of RNA by in Vitro Run-Off Transcription. Construction of clone G11 has been described elsewhere (Guo et al., 1993). G11 DNA (see Figure 1) was linearized by digestion with *Ssp*I, extracted with 1:1 phenol/CIA (CIA = chloroform/isoamyl alcohol, 24:1), precipitated with ethanol, and dissolved in water. Standard T7 transcription reactions (10 μL) contained approximately 1 μg of DNA, 40 mM Tris-HCl, pH 8.0, 125 mM NaCl, 2 mM spermidine, 4 mM dithiothreitol, 1 mM of each NTP, 0.5–2.0 μCi of [α-^{32}P]-GTP (3000 Ci/mmol), 12–20 units of RNAguard (Pharmacia), 25 units of T7 RNA polymerase (Bethesda Research Laboratories), and 8 mM $MgCl_2$. In order to obtain uncleaved Pre RNA, the concentration of $MgCl_2$ was lowered to 3 mM (see Figure 1B,C). Reactions were incubated for 60 min at 37 °C, extracted once with phenol/CIA and once with CIA, precipitated with ethanol, washed with 70% ethanol, reprecipitated, washed again, and dissolved in water. We refer to the resulting Pre RNA as "crude" Pre RNA to distinguish it from gel-purified Pre RNA. Crude Pre RNA was used for all experiments except those indicated in Figure 2A, in which gel-purified Pre RNA was used. Gel-purified Pre RNA was obtained by electrophoresis of crude Pre RNA in a 4% polyacrylamide/8.3 M urea 1× TBE gel and elution in water overnight at 4 °C. Eluted RNA was filtered through a 0.45-μm cellulose acetate membrane to remove residual polyacrylamide, precipitated with ethanol, washed with 70% ethanol, and dissolved in water.

Self-Cleavage Reactions. Appropriate reaction solutions and RNA (in water) were preincubated separately for 5 min at the reaction temperature. Aliquots were then mixed to initiate the reaction and obtain the ionic concentrations specified in the figure legends. Typical reactions contained approximately 0.4 μg of RNA in a 36-μL final volume ([RNA] ≃ 200 nM). Aliquots (4 μL) were removed after 0, 2, 4, 6, 8, 10, 30, and 60 min of incubation and mixed with 8 μL of

† This research was supported by an operating grant and a scientist award from the Medical Research Council of Canada

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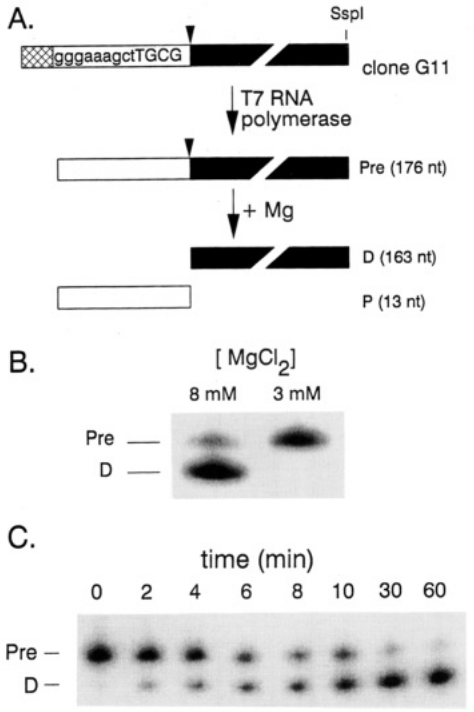

FIGURE 1: Self-cleavage of Pre RNA. (A) Sequence organization of clone G11 and the products of transcription and self-cleavage. G11 DNA was linearized at the *Ssp*I site at position 783, numbered as in Saville and Collins (1990). Pre RNA (176 nt) was synthesized by in vitro transcription using T7 RNA polymerase (Milligan & Uhlenbeck, 1989; see Materials and Methods). Self-cleavage of Pre produces P (13 nt) and D (163 nt) RNAs. Vector nucleotides are indicated in lower case letters. The arrow marks the site of self-cleavage after G620. The hatched box indicates the T7 promoter. (B) Pre RNA was synthesized in transcription buffer containing 8 or 3 mM $MgCl_2$ in the presence of [α-^{32}P]GTP, electrophoresed in a denaturing 4% polyacrylamide gel, and detected with a PhosphorImager screen (see Materials and Methods for details). (C) Pre RNA synthesized in transcription buffer containing 3 mM $MgCl_2$ was incubated in 40 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, and 2 mM spermidine at 37 °C for the times indicated, separated by denaturing gel electrophoresis, and detected with a PhosphorImager screen.

stop dye (80% v/v formamide, 0.5× TBE, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue). Slower reactions performed with low concentrations of magnesium chloride (Figure 3) were extended to 4 h. RNAs were separated by electrophoresis on gels containing 4% polyacrylamide, 8.3 M urea, and 1× TBE (BRL V-16 apparatus), covered with plastic wrap, and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). Radioactivity was quantitated with the ImageQuant 3.0 software. Pre RNA contains [α^{32}P]-guanosine at 52 positions: after cleavage, 46 guanosines are in the 163 nt D RNA and 6 are in P RNA. Data were plotted as fraction cleaved vs. time. Fraction cleaved was calculated as D/(Pre + D) and not corrected for the loss of the small amount of radioactivity in P, which was routinely run off these gels to facilitate quantitation by increasing separation between Pre and D.

RESULTS AND DISCUSSION

As is typical of other ribozymes, we have found that only a small fraction of the native 881 nt length of VS RNA is required for self-cleavage (Guo et al., 1993). For the experiments presented here, we used a subclone of VS DNA called G11 (Figure 1A). In vitro transcripts of this clone begin with three guanosines to maximize the yield of RNA by T7 RNA polymerase transcription, followed by a unique *Hin*dIII restriction site to facilitate future subcloning and four VS nucleotides upstream of the cleavage site. When the template DNA is linearized at a convenient *Ssp*I restriction site, a 176 nt transcript (Pre) can be synthesized which cleaves to produce a 13 nucleotide upstream cleavage product (P) and a 163 nt downstream product (D).

Synthesis and Properties of Pre RNA. When Pre RNA was synthesized by in vitro transcription in our standard T7 RNA polymerase buffer, which contains 8 mM $MgCl_2$ and 1 mM of each nucleoside triphosphate (NTP), 50–95% of the RNA cleaved during the transcription reaction (Figure 1B). The remaining uncleaved precursor RNA could be separated by gel electrophoresis and eluted to provide a source of precursor for kinetic studies; however, the amount of precursor obtained was variable and inconveninently small. In other experiments (not shown), we discovered that transcription in the presence of high concentrations (4 mM) of any of several NTPs completely inhibited self-cleavage. No inhibition was observed when the same concentrations of the corresponding bases or nucleoside monophosphates were added to standard transcription reactions, and complete inhibition could alternatively be obtained by decreasing the concentration of $MgCl_2$ in the standard transcription reaction to 2 or 3 mM (Figure 1B). These results suggested that inhibition of self-cleavage was due to chelation of some of the magnesium by the nucleoside triphosphates; evidently, T7 RNA polymerase can function at a magnesium concentration lower than that required for Pre RNA self-cleavage.

When Pre RNA was synthesized in a transcription reaction that contained 3 mM $MgCl_2$ and 1 mM of each NTP, and then incubated in a solution that allowed self-cleavage, it cleaved essentially to completion during a 1-h incubation [Figure 1C; in early experiments we used the standard T7 transcription solution (see Materials and Methods) for the self-cleavage reaction (data not shown); the time course shown in Figure 1C used a solution with fewer components (see legend)]. These results suggest that the low magnesium concentration during synthesis did not have a significant effect on the fidelity of transcription or on the ability of the RNA to adopt a functional conformation during the subsequent incubation in self-cleavage buffer.

Purification of crude Pre RNA by denaturing gel electrophoresis had little, if any, effect on the kinetics of self-cleavage when the purified RNA was subsequently incubated in self-cleavage buffer (Figure 2A). Consequently, in order to obtain Pre RNA essentially free of cleavage products, we used a transcription solution containing 3 mM $MgCl_2$ and omitted gel purification.

Some other ribozymes require heating and snap cooling to convert the RNA into an active form (Forster & Symons, 1987b; Wu & Lai, 1990). Such treatment had no effect on either the extent or the rate of cleavage of Pre RNA (compare Figure 2B, in which RNAs were heated and cooled, with Figure 2A, in which they were not). In most of our experiments, 90% to >99% of Pre RNA cleaved during a 1-h time course, indicating that little, if any, is in a stable noncleavable conformation. Consequently, we did not heat and cool the RNA for most of the experiments described below.

Cleavage of Pre RNA Is an Intramolecular Reaction. With some other ribozymes, apparent "self-cleavage" has actually been found to occur in a bimolecular complex of two identical molecules and thus should be thought of as an intermolecular "trans-cleavage" reaction. In such reactions the initial rate of cleavage is strongly affected by concentration of the RNA and would be expected to exhibit second-order kinetics (Forster

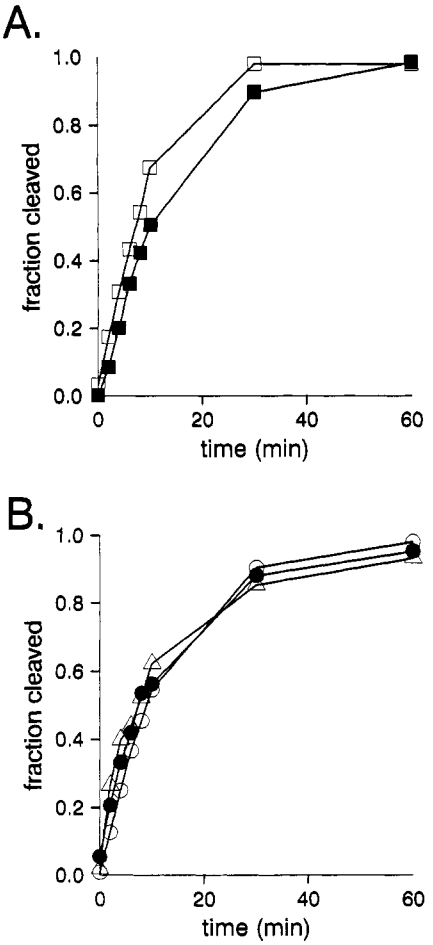

FIGURE 2: Kinetics of Pre RNA self-cleavage. (A) Time course of self-cleavage of crude or gel-purified Pre RNAs. Crude Pre RNA was synthesized in a buffer containing 3 mM $MgCl_2$ to minimize self-cleavage during transcription. An aliquot was purified by denaturing gel electophoresis. Each RNA was incubated as in Figure 1C. Aliquots were removed at various times during the incubation and analyzed by denaturing gel electrophoresis and quantitated. (Open squares) Gel-purified Pre; (filled squares) crude Pre. See Materials and Methods for details. (B) Lack of effect of RNA concentration on the rate of self-cleavage. Crude Pre RNA at approximately 250 (open circles), 25 (filled circles), or 2.5 (open triangles) nM was heated to 95 °C for 3 min, cooled on ice, and incubated as in Figure 1C.

et al., 1988; Cremisi et al., 1992). In other work, we have found that certain fragments of VS RNA (different than those used in the current paper) which lack a self-cleavage site can cleave a separate RNA in a true enzymatic manner (H. Guo and R. A. C, unpublished observations). Similar trans reactions have also been demonstrated with other ribozymes (Uhlenbeck, 1987; Feldstein et al., 1989; Hampel & Tritz, 1989; Haseloff & Gerlach, 1989; Perrotta & Been, 1992; Branch & Robertson, 1991). Thus, at least in principle, the Pre RNA could act in trans. However, we found that the initial rate of cleavage of Pre RNA was not substantially different over the range examined (from approximately 2.5 to 250 nM, Figure 2B). Heating and snap cooling had no effect on the initial rate of cleavage at even low RNA concentrations. This suggests that bimolecular complexes had not formed during sample preparation or during the cleavage reaction. These observations suggest that bimolecular complexes are not involved in self-cleavage, unless they form very rapidly even at the lowest RNA concentrations examined.

Determination of Optimal Reaction Conditions. A substantial fraction of Pre RNA cleaves during transcription in the standard T7 RNA polymerase reaction (Figure 1B). In order to determine which components of the solution affect the cleavage rate, we have investigated the effect of several variables that would be expected to affect RNA structure and that have been found to affect the cleavage rates of ribozymes derived from other RNAs (Figures 3–6).

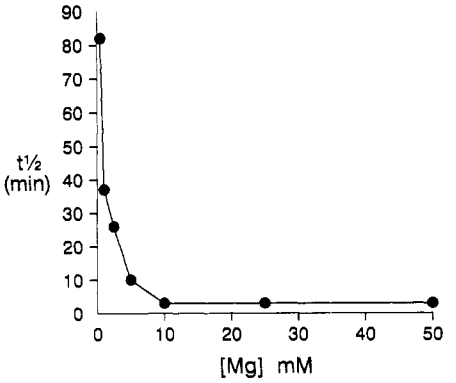

FIGURE 3: Cleavage as a function of concentration of magnesium chloride. Pre RNA was incubated at 37 °C in 40 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM spermidine, and $MgCl_2$ concentrations ranging from 0 to 50 mM. No cleavage was observed in the absence of magnesium.

No self-cleavage of Pre RNA was observed in the absence of a divalent cation, and the rate of cleavage increased with increasing concentration of $MgCl_2$, reaching a maximum at around 10 mM (Figure 3). Manganese and calcium can substitute for magnesium but are not as effective. When these divalent cations replaced $MgCl_2$ in our standard cleavage solution (which also contained 40 mM Tris-HCl, pH 8.0, 50 mM KCl, and 2 mM spermidine), the most rapid self-cleavage observed over a range of divalent cation concentrations (0.5–25 mM) had $t_{1/2}$ of around 60 min with $MnCl_2$ or 75 min with $CaCl_2$ (data not shown), compared with 3 min for $MgCl_2$ (Figure 3). We chose a magnesium chloride concentration of 5 mM for subsequent experiments, because the cleavage rate under these conditions ($t_{1/2} \simeq 10$ min) would allow easy measurement of rate changes as a function of changes in other reaction parameters (Figures 4–6).

Self-cleavage occurs with 5 mM magnesium as the only cation (Figure 4). Spermidine enhances the reaction rate (Figure 4A) but cannot substitute for magnesium (compare with Figure 3, where no cleavage was detected in the absence of $MgCl_2$ even though that reaction contained 2 mM spermidine). Monovalent cations also stimulate the reaction substantially: results for potassium chloride are shown in Figure 4B. Ammonium also stimulates; sodium is less effective (data not shown). Stimulation by spermidine and KCl is not additive: in the presence of 6 mM KCl, little, if any, further stimulation by spermidine was observed (Figure 4C). These observations are consistent with spermidine and potassium acting as structural counterions, which facilitate folding of the RNA. The additional requirement for a divalent cation implies that it may perform a specific role in folding of the RNA and/or that it is directly involved in the cleavage mechanism.

The optimum temperature for self-cleavage is 40–50 °C, and significant activity is still present at 60 °C (Figure 5A). At temperatures of 70 °C and above, substantial degradation of the RNA was observed (data not shown). This is typical of the range of temperatures at which many other ribozymes are active, although some ribozymes derived from HDV are active at even higher temperatures (Rosenstein & Been, 1990; Wu & Lai, 1990).

Little, if any, effect of pH was observed on the rate or extent of Pre RNA cleavage between pH 5.5 and 8.9 (Figure

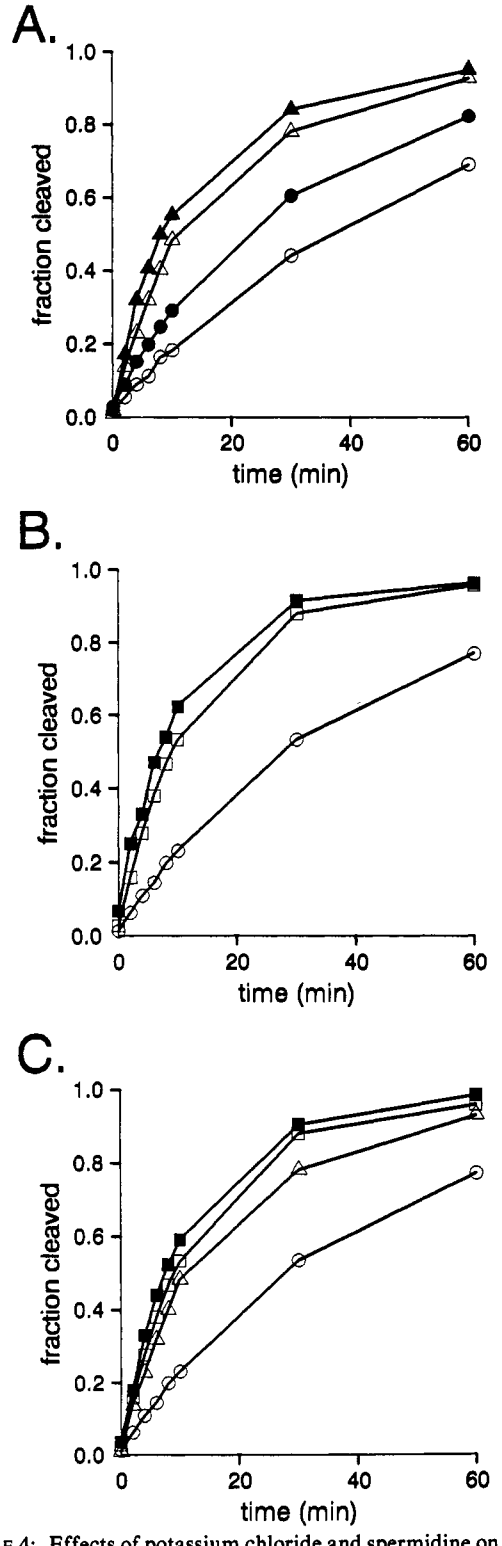

FIGURE 4: Effects of potassium chloride and spermidine on the rate of self-cleavage. (A) Spermidine concentration: (filled triangles) 5 mM; (open triangles) 2 mM; (filled circles) 0.5 mM; (open circles) 0 mM. (B) KCl concentration: (filled squares) 50 mM; (open squares) 6 mM; (open circles) 0 mM. (C) (filled squares) 6 mM KCl + 2 mM spermidine; (open squares) 6 mM KCl; (open triangles) 2 mM spermidine; (open circles) no KCl, no spermidine. Pre RNA was incubated at 37 °C in 40 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and the indicated concentration of KCl and/or spermidine.

5B). At pH 5.0 the reaction was much slower, possibly as a result of base protonation (Puglisi et al., 1990; Santa Lucia et al., 1991), which could lead to significant changes in RNA conformation. Similar cleavage rates were observed whether the Pre RNA was gel-purified or not, or whether or not

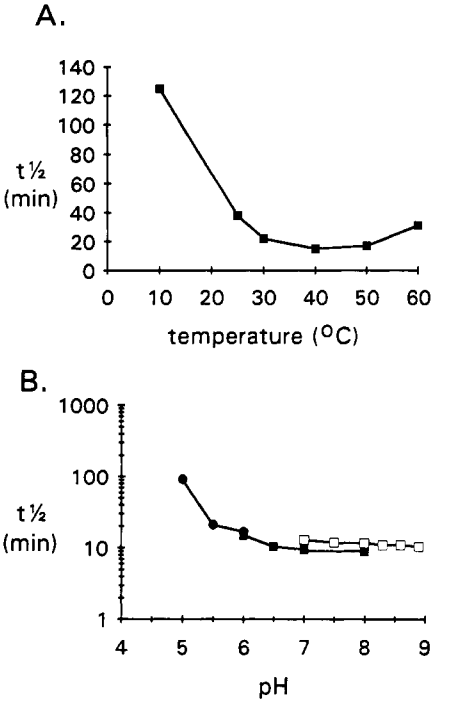

FIGURE 5: Effect of temperature and pH on the rate of self-cleavage. (A) Pre RNA was incubated as in Figure 1C at each of the temperatures indicated. (B) Pre RNA was incubated as in Figure 1C except that different buffers were used to span the range of pH examined: (filled circles) 40 mM MES; (filled squares) 44 mM Tris/16.5 mM PIPES; (open squares) 40 mM Tris.

spermidine was present during the reaction (data not shown). A lack of effect of pH has also been reported for self-cleavage of a HDV ribozyme (Wu & Lai, 1990) and for trans-cleavage (bimolecular) reactions catalyzed by RNase P (Guerrier-Takada et al., 1986) and by a hammerhead ribozyme (Uhlenbeck, 1987). However, recent experiments in which the actual cleavage step was shown to be rate-limiting have revealed an approximately 10-fold rate enhancement per unit increase in pH, consistent with a direct involvement of hydroxide in the hammerhead and RNase P reaction mechanisms (S. A. Dahm and O. C. Unlenbeck, personal communication, and D. Smith and N. Pace, personal communication). The effect of pH on *self*-cleavage of hammerhead ribozymes has not been investigated in detail. Such an intramolecular cleavage reaction, by definition a single-turnover event, might be expected to show a pH effect comparable to that observed under single-turnover conditions with the intermolecular trans-cleavage reaction, assuming that both proceed by the same mechanism.

The lack of effect of RNA concentration on the rate of VS RNA cleavage (Figure 2B) strongly suggests that this is indeed an intramolecular, i.e., single-turnover, reaction. Thus, steps such as product release following the cleavage event would not affect the apparent cleavage rate. The lack of effect of pH between pH 5.5 and 8.9 suggests either that hydroxide is not directly involved in the cleavage step or that some step prior to the actual cleavage event, for example, a conformation change in the RNA, is rate-limiting.

Effects of Denaturants. VS and HDV ribozymes are the only ribozymes currently known that require for activity only a single nucleotide upstream of the cleavage site (Perrotta & Been, 1990; Guo et al., 1993). Both potentially form a GC-rich helix immediately downstream of the cleavage site; however, beyond these limited similarities, the VS and HDV RNAs do not share obvious structural features. One of the

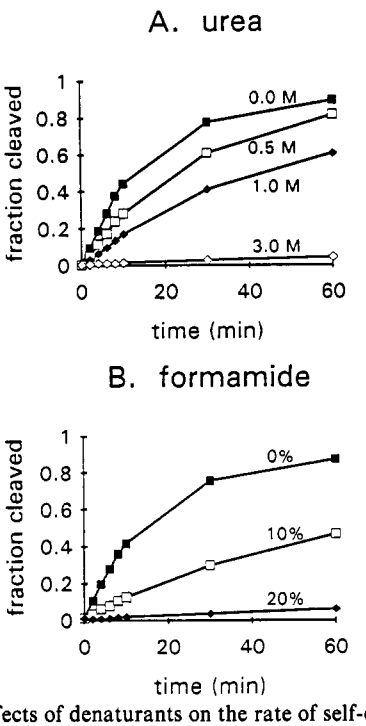

FIGURE 6: Effects of denaturants on the rate of self-cleavage. Pre RNA was incubated at 37 °C in 40 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 2 mM spermidine, and the indicated concentrations of urea or formamide.

distinctive functional features of HDV ribozymes is their activity in the presence of high concentrations of formamide or urea (Rosenstein & Been, 1990; Wu & Lai, 1990; Smith & Gottleib, 1991). In order to determine if the limited structural similarity between VS and HDV was reflected in functional properties, we examined the effect of a range of concentratioins of these denaturants on the cleavage activity of Pre RNA. The data in Figure 6 show that even low concentrations of denaturants cause detectable inhibition, and >90% inhibition is observed with only 10–20% formamide or 1.6 M urea. Thus, VS and HDV differ in function and presumably in structure.

ACKNOWLEDGMENT

We thank Debbie Field for comments on the manuscript and Olke Unlenbeck and Marty Fedor for discussions about pH effects.

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