Biochemistry **1998,** *37,* 6476−6484

Spermine Switches a *Neurospora* VS Ribozyme from Slow Cis Cleavage to Fast Trans Cleavage[†]

Joan E. Olive and Richard A. Collins*

Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Received October 20, 1997; Revised Manuscript Received February 16, 1998

ABSTRACT: In keeping with the known role of polyamines as counterions in RNA folding, we have found that concentrations of spermine as low as 1 μM facilitate first-order Cis cleavage and decrease the concentration of magnesium required for optimal cleavage of the VS ribozyme. Surprisingly, under certain experimental conditions, cleavage reactions at concentrations of spermine above about 20 μM were not first-order. At 100 μM spermine, about half of the RNA cleaved in an initial very fast burst, $k \geq 5$ min^{-1}, about 100-fold faster than under our previously optimized conditions; the remainder of the RNA cleaved more slowly. The extent of the burst and the initial rate of cleavage were proportional to RNA concentration, suggesting that the fast phase was due to intermolecular trans cleavage involving two or more RNAs. This inference of trans cleavage was confirmed by demonstrating spermine-dependent trans cleavage of particular combinations of mutant RNAs that were each incapable of cis cleavage. The experimental conditions required to switch the VS ribozyme into trans cleavage mode are quite stringent. The RNA must be preincubated with an adequate concentration of spermine at very low ionic strength near neutral pH. Concentrations of buffers and salts typically used in in vitro studies of ribozymes, including those used in our previous characterization of the VS ribozyme, are sufficiently high that they prevent or reverse the trans cleaving RNA conformation. The ability to switch cleavage modes from cis to trans provides an experimental system to study different active conformations of VS RNA, as well as to investigate the functional consequences of polyamine−RNA interactions.

Several small ribozymes, termed hammerhead, hairpin, hepatitis delta virus, and *Neurospora* VS, have been identified in naturally occurring RNAs. Their biological role is to perform self-cleavage reactions to release monomer-length RNAs from multimeric transcripts or replication intermediates (for review, see ref *1*). These ribozymes have also provided excellent model systems with which to study RNA folding and catalysis. Characterization of these cleavage reactions in vitro has shown that most proceed via intramolecular cis cleavage, although intermolecular trans cleavage occurs in certain hammerhead ribozymes in which one helix is too short to be stable in an intramolecular structure (*2*). All of these ribozymes have also been redesigned to function in trans by separating the cleavage site and the catalytic core onto separate RNA transcripts (*3*−*8*). In general, cleavage of a given RNA proceeds either in cis or in trans as a consequence of the nature or design of the RNA(s) involved.

Catalytic activity depends on tertiary folding of the RNA that positions functional groups and/or bound metal ions in the proper orientation with respect to the scissile phosphodiester bond. For most ribozymes, divalent metal ions (typically magnesium) can perform both the folding and catalytic functions (for review, see refs *9*, *10*). Other oligocations, such as the polyamines spermine and spermidine, can partially substitute for metal ions in facilitating RNA folding by bridging the negatively charged phosphate backbones via interactions with the amino groups on the polyamine (*11*). By stabilizing folded RNA structures, polyamines reduce the concentration of magnesium required for catalysis and can even suppress the cleavage deficiencies of certain mutant ribozymes (*12*). Slow polyamine-enhanced ribozyme-catalyzed cleavage by hairpin and hammerhead ribozymes has been observed in the absence of magnesium, although it has not been determined how the mechanism of such cleavage relates to the much faster reactions in the presence of magnesium (*13*, *14*).

We report here an unexpected effect of spermine on the cleavage properties of the *Neurospora* VS ribozyme. Although low concentrations of spermine facilitate cis cleavage, as expected, concentrations above about 20 μM can switch the RNA into a conformation that cleaves very rapidly in trans.

EXPERIMENTAL PROCEDURES

Preparation of RNAs. Precursor RNAs were synthesized by in vitro transcription using T7 RNA polymerase in the presence of [α-^{32}P]GTP from clone G11 (or its site-directed mutants) which had been digested with *Ssp*I, as described previously (*15*). The sequence and proposed secondary structure of G11 Pre RNA are diagrammed in Figure 1. RNAs were purified by denaturing gel electrophoresis on

---

[†] Supported by the Medical Research Council of Canada. R.A.C. is a Fellow of the Canadian Institute for Advanced Research Program in Evolutionary Biology.

* Author to whom correspondence should be addressed at Department of Molecular and Medical Genetics, University of Toronto, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8. Phone: (416) 978-3541. Fax: (416) 978-6885. E-mail: rick.collins@utoronto.ca.

S0006-2960(97)02584-1 CCC: $15.00

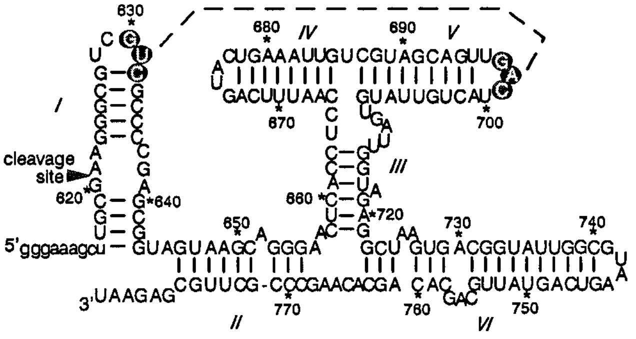


FIGURE 1: Secondary structure model of G11 Pre RNA. Bases and helices are numbered as in (*25*). VS and vector nucleotides are indicated in upper- and lowercase, respectively. Cleavage at the site indicated by the arrowhead yields promoter-proximal and -distal fragments G11 P and G11 D. Bases involved in the pseudoknot interaction between stem-loops I and V are highlighted in black and joined by a dashed line.

4% polyacrylamide gels containing 8.3 M urea and 1× TBE (1× TBE = 135 mM Tris, 45 mM boric acid, 2.5 mM EDTA), eluted in water at 65 °C for 30 min or at 4 °C overnight, filtered through an Acrodisk (Gelman Scientific), precipitated with ethanol twice in the presence of 0.3 M sodium acetate, and stored at −20 °C in water.

Cleavage Reactions. Unless otherwise specified, the RNA concentration in cleavage reactions was approximately 50 nM. For cleavage reactions using preincubated RNAs, aliquots of RNA were adjusted to 5 mM Tris-HCl pH 7.5, 5 μM EDTA and preincubated with spermine-HCl (Sigma Chemical Co., St. Louis, MO) to give the final concentrations indicated in figure legends in four-fifths of the final reaction volume for 30 min at 37 °C. Cleavage reactions were started by addition of one-fifth volume of pre-warmed standard buffer (final concentrations: 40 mM Tris-HCl pH 8.0, 50 mM KCl, 25 mM $MgCl_2$) or $MgCl_2$ to final concentrations indicated in the figures. Reactions were stopped by addition of two volumes of formamide loading solution (80% formamide, 10 mM EDTA, 0.5× TBE, 0.05% each xylene cyanol and bromophenol blue). Samples were resolved by electrophoresis on 4% polyacrylamide/ 8.3 M urea gels. Band intensities corresponding to Pre and D were determined using a PhosphorImager and expressed as fraction cleaved = D/(Pre + D) or, for first-order plots, fraction of Pre remaining, *f*(pre) = 1 − fraction cleaved. Whenever appropriate, cleavage rate constants were determined from the slopes of first-order plots. Initial rates of the rapid spermine-enhanced reactions were estimated as $k = 0.693/t_{1/2}$, where $t_{1/2}$ was extrapolated from determinations of the fraction of precursor RNA remaining in aliquots taken during the first few seconds of the reaction (see Figure 6C for an example).

RESULTS

Preincubation with Spermine Induces Very Rapid Cleavage. In our previous characterization of G11 RNA, which contains the minimal contiguous self-cleaving region of VS RNA flanked by a few nonessential nucleotides (Figure 1), we found that optimal cleavage occurred in concentrations of buffer (25 to 50 mM), Mg^{2+} (2 to 25 mM), and monovalent salt (10 to 50 mM) that are similar to the reaction conditions used in the in vitro study of many other ribozymes (*16*). Addition of spermine to our standard buffer (40 mM Tris pH 8.0, 50 mM KCl, 25 mM $MgCl_2$) had essentially no effect on the cleavage rate (Figure 2A). This was not surprising, considering previous observations which showed that either KCl or spermidine (spermine was not examined previously) were effective counterions, and that their effects on cleavage rate and magnesium optimum were independent rather than additive (*16*). Presumably the concentrations of KCl and $MgCl_2$ in our standard buffer provided a saturating concentration of counterions to allow proper RNA folding, so no additional effects of spermine were observed under these conditions.

In contrast to the expected results described above, when G11 RNA was preincubated with spermine before addition of standard buffer about half of the RNA cleaved in a very rapid burst within a few seconds after addition of standard buffer (Figure 2B). The remaining RNA cleaved at a slower rate that was superficially similar to the rate observed in standard buffer alone; however, closer examination showed that this post-burst cleavage was not a simple first-order reaction (see below). The enhancement by spermine was only observed if the RNA was preincubated with spermine before addition of the remaining components to initiate the cleavage reaction (Figure 2C). These observations suggested that preincubation with spermine favors a conformation of the RNA that is capable of very rapid cleavage. That only a fraction of the RNA cleaves at the fast rate suggests either that only a fraction is in the fast conformation under these conditions or that this conformation is rapidly disrupted by standard concentrations of buffer, salt, and/or magnesium.

To investigate the nature of the extremely rapid cleavage observed after preincubation with spermine, we studied the individual and combined effects of varying the concentrations of RNA, $MgCl_2$, and other cations on the kinetics of the cleavage reaction in a minimal buffer containing 5 mM Tris pH 7.5 and 5 μM EDTA. We examined the effects of preincubation with any of several cations or polyamines on the observed cleavage rate of reactions initiated with $MgCl_2$ to a concentration of 2.5 mM, which in the absence of other cations supports only very slow cleavage (Figure 3, and data not shown). Consistent with our previous characterization using standard buffer, KCl or spermidine were effective counterions. Sodium chloride and the smaller polyamines putrescine and cadaverine had essentially no effect on the rate of cleavage. Spermine, which we had not examined in our previously published characterization, showed the greatest enhancement of the cleavage reaction.

Figure 4A shows the results of cleavage reactions with different concentrations of Mg^{2+} as the only cation (i.e., no spermine added). As observed in reactions using standard buffer (Figure 2A), ≥70% of the RNA cleaved with first-order kinetics. In contrast, Figure 4, parts B−D, shows that when the RNA was preincubated with 0.1 mM spermine, the cleavage curves observed upon addition of $MgCl_2$ were quite different from the typical first-order reactions shown in Figure 4A. At very low Mg^{2+} concentration, 0.001 to 0.05 mM, although the first 30% to 50% of the cleavage reaction appeared to fit reasonably well to a first-order analysis, the reactions slowed progressively as cleavage proceeded (Figure 4B). The biphasic nature of the reaction was more evident at higher Mg^{2+} concentration; above $\sim$0.2 mM Mg^{2+}, a fraction of the RNA cleaved in a fast burst

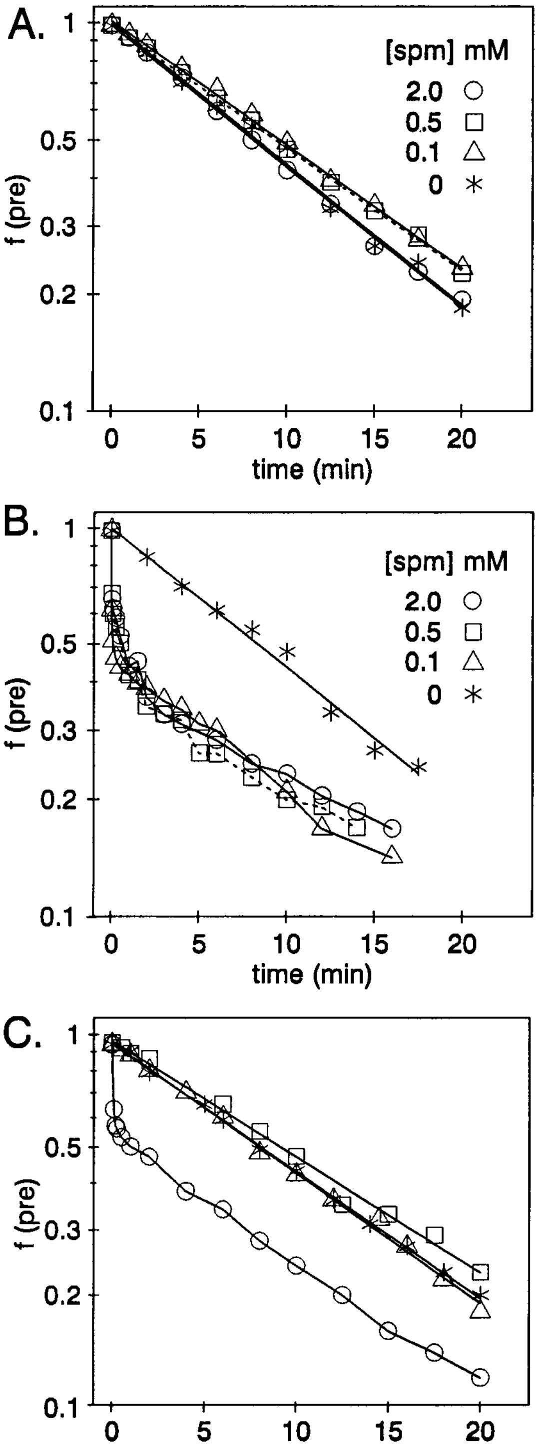


FIGURE 2: Spermine-enhanced rapid cleavage requires preincubation of G11 Pre RNA with spermine. (A) RNA was preincubated for 30 min at 37 °C in 5 mM Tris-HCl pH 7.5, 5 μM EDTA in the absence of spermine; reactions were started by addition of standard buffer (final concentrations: 40 mM Tris-HCl pH 8.0, 50 mM KCl, 25 mM $MgCl_2$) plus the indicated final concentrations of spermine. (B) RNA was preincubated as above but in the presence of the indicated concentrations of spermine; reactions were started by addition of standard buffer. (C) RNA was preincubated as above in the presence (circles) or absence (triangles) of 0.1 mM spermine; cleavage reactions were started by addition of standard buffer. Control RNAs were not preincubated, and reactions were started by addition of standard buffer with (squares) or without (asterisks) 0.1 mM spermine. Data were fit to a first-order equation, except for reactions that showed a burst of cleavage where the line simply connects the data points.

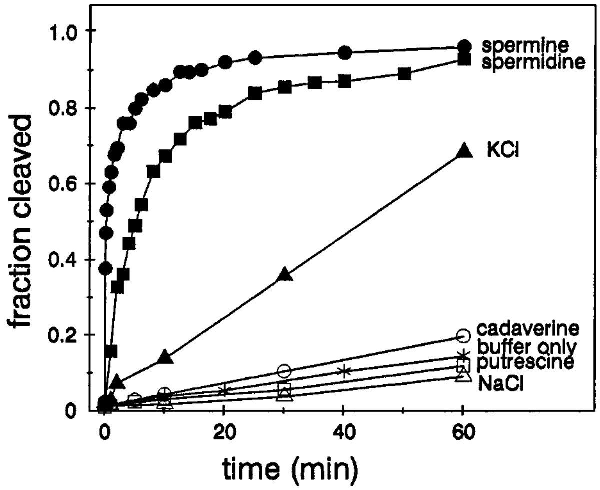


FIGURE 3: Effects of cations on the cleavage reaction. RNAs were preincubated as in Figure 2 in 5 mM Tris-HCl pH 7.5, 5 μM EDTA only or including 0.1 mM spermine, 5 mM spermidine, 5 mM cadaverine, 5 mM putrescine, 1 M NaCl or 1 M KCl. Reactions were started by addition of $MgCl_2$ to 2.5 mM.

(Figure 4C, note the different *x*-axis scales in each part of Figure 4); at 25 mM Mg^{2+}, almost half of the RNA cleaved in the first few seconds after addition of Mg^{2+} (Figure 4D). The rate of cleavage of the remaining RNA was substantially slower and gradually decreased as the reaction proceeded, similar to the cleavage curves observed with spermine-preincubated RNA using standard buffer (see Figure 2B).

A plot of the initial rates of cleavage vs $MgCl_2$ concentration for the RNA preincubated with or without spermine is shown in Figure 5. Compared to the rate vs [$MgCl_2$] curve of the Mg^{2+}-only reactions (filled circles), the RNA preincubated with spermine (open circles) showed detectable cleavage activity at 100-fold lower Mg^{2+} concentration, ~0.005 mM, and maximal activity, >5 min^{-1}, was achieved at only a few millimolar Mg^{2+}. The initial cleavage rates at Mg^{2+} concentration above a few millimolar are minimum estimates and were too fast to measure accurately by manual pipetting. This rapid cleavage rate is about 100-fold higher than in the absence of spermine, even at saturating Mg^{2+} (Figure 5, filled circles) or in our previously determined optimal conditions using KCl or spermidine as counterions (*16*). The magnitude of the effect of spermine on the cleavage rate of VS RNA is far greater than has been reported for other ribozymes.

Spermine Induces Intermolecular Trans Cleavage. The details of the cleavage reaction following preincubation of RNA with spermine are more complex than implied from the data above, in which the spermine concentration was fixed at 100 μM. When cleavage reactions were performed at lower spermine concentrations, below about 5 μM, and the reaction was initiated by addition of $MgCl_2$ to 2.5 mM, cleavage exhibited first-order kinetics, as expected for cis cleavage. First-order cleavage was observed regardless of whether the RNA was preincubated with spermine or the spermine was added with the $MgCl_2$ to start the reaction (Figure 6A,B; summarized in Figure 7).

In contrast, at higher spermine concentrations (above about 20 μM) cleavage was not a simple first-order reaction (Figure 6A,B), nor did the data fit well to an analysis assuming two populations of RNA with different first-order rates, as has

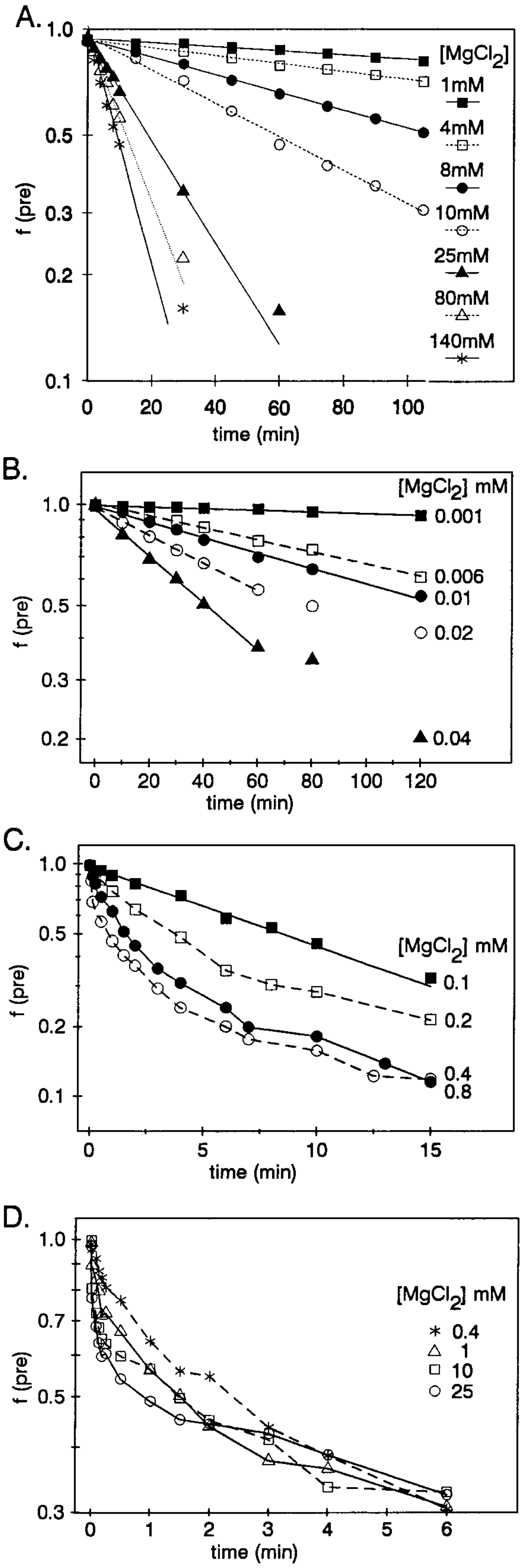


FIGURE 4: Dependence of the cleavage reaction on $MgCl_2$ concentration. RNAs were preincubated as in Figure 2 in the absence of spermine (panel A) or in the presence of 0.1 mM spermine (panels B−D). Reactions were started by addition of $MgCl_2$ to the final concentrations indicated. Data were fit to a first-order equation, except for reactions that show a burst of cleavage where the line simply connects the data points.

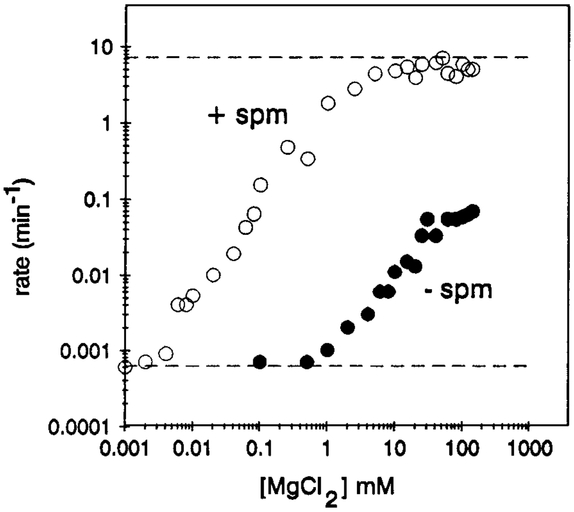
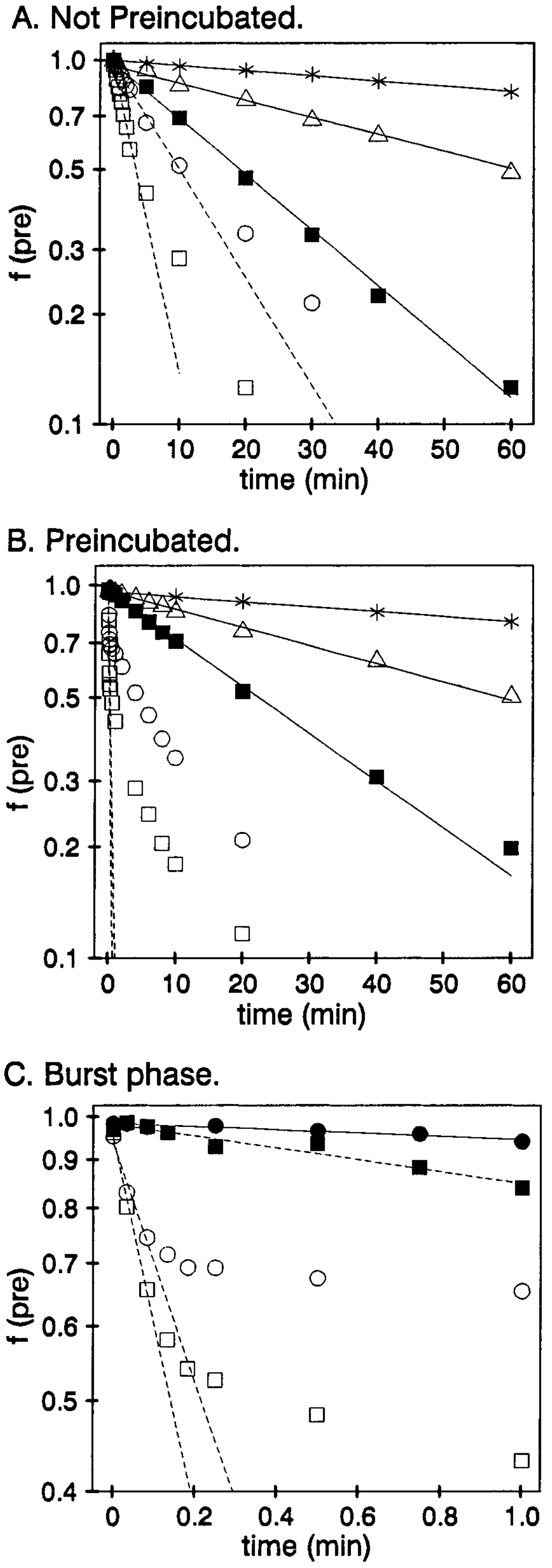


FIGURE 5: Spermine increases the rate and decreases the magnesium optimum of the cleavage reaction. Cleavage rates were estimated from experiments in Figure 4 (and data not shown using other concentrations of magnesium) in the presence or absence of 0.1 mM spermine. Dashed lines indicate the upper and lower limits of cleavage rates that are measurable by our assay.

been observed with some other ribozymes (e.g. ref *17*; data not shown). The biphasic nature of the curves at higher spermine concentration was most noticeable when the RNA was preincubated with spermine (Figure 6B), as was also observed in reactions initiated with standard buffer (Figure 2B,C). A fraction of the RNA cleaved in a remarkably fast burst, at least 4 min^{-1} (Figure 6B,C). The fraction of RNA that cleaved in the burst phase increased with increasing spermine concentration until, at optimal spermine concentration (about 100 μM), about one-third of the RNA cleaved during the burst (Figure 6C, and data not shown). Figure 7 shows the initial rates of cleavage as a function of spermine concentration for RNA preincubated with spermine (circles) compared to the rates of reactions initiated by adding spermine and $MgCl_2$ simultaneously (squares). These data show that spermine concentrations below about 10 μM increase the cleavage rate independent of preincubation, consistent with the known role of polyamines in facilitating folding of RNA. Higher spermine concentrations continue to increase the rate when added with $MgCl_2$; however, preincubation of the RNA with higher concentration of spermine leads to an exceptionally rapid burst of cleavage.

The biphasic cleavage curves and the progressive decrease in cleavage rate as the reaction proceeded suggested that cleavage in spermine concentration above about 20 μM might be a second-order (or higher) reaction in which one G11 RNA cleaves another in trans. This hypothesis predicted that the initial cleavage rate at low spermine concentration would be independent of RNA concentration, but at high spermine concentration cleavage rate would be proportional to RNA concentration. Figure 8 shows that this is indeed the case. At 5 μM spermine, the cleavage curves were first-order, showing no difference in rate at different concentration of RNA. In contrast, at 100 μM spermine the extent of the burst and the initial rate of cleavage increased with increasing RNA concentration reaching saturation around 100 nM RNA (Figure 8B, and data not shown). These observations indicate that low spermine concentration facilitates cis cleavage, while higher spermine concentration favors forma-


A. Not Preincubated.

B. Preincubated.

C. Burst phase.

FIGURE 6: Low and high concentrations of spermine affect the reaction differently. A. RNA was preincubated for 30 min at 37 °C in 5 mM Tris-HCl pH 7.5, 5 μM EDTA in the absence of spermine; reactions were started by simultaneous addition of $MgCl_2$ to 2.5 mM and spermine to 0.1 μM (asterisks), 1.0 μM (triangles), 5 μM (filled squares), 40 μm (circles), or 100 μM (open squares). (B) RNA was preincubated as above, but in the presence of the concentrations of spermine indicated in (A); reactions were started by addition of $MgCl_2$ to 2.5 mM. (C) Expanded plot of the first 1 min of the cleavage reactions of RNAs preincubated (open symbols) or not (filled symbols) with 40 μM (circles) or 100 μM (squares) spermine.

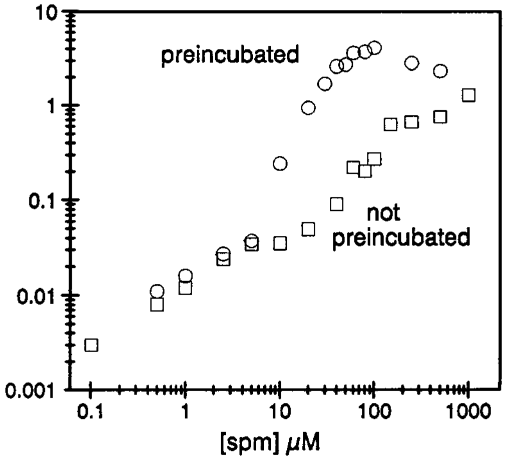


FIGURE 7: Effects of spermine concentration and preincubation on the rate of the cleavage reaction. Cleavage rates were estimated from experiments in Figure 6 (and data not shown using other concentrations of spermine) in which the indicated concentration of spermine was preincubated with the RNA (circles) or added with $MgCl_2$ to start the reaction (squares).

tion of an intermolecular complex capable of rapid trans cleavage.

Trans Cleavage of Mutant RNAs. Independent support for the proposal that spermine induces trans cleavage was obtained by using mutants that were incapable of cis cleavage due to disruption of a pseudoknot involving nucleotides in loops I and V (diagrammed in Figure 1). We showed previously that base substitution mutations that disrupted the loop-loop pseudoknot showed severely decreased activity, but certain double mutants in loops I and V that restored base pairing partially restored activity (*18*). Our working model is that the pseudoknot is one of the interactions that folds stem-loop I into the active structure so that the cleavage site on the 5′ side of stem I can be cleaved. Even though pseudoknot mutants are inactive for cis cleavage, we reasoned that spermine might be able to induce the formation of an active intermolecular complex between two mutant RNAs, one with a single mutations in loop I, the other with a complementary mutation in loop V, as diagrammed in Figure 9A. Although neither single mutant is capable of intramolecular cis cleavage, two RNAs might form an intermolecular version of the pseudoknot in which the loop I mutant RNA would be cleaved in trans by pairing with the complementary mutant loop V RNA; the wild-type stem-loop I of any loop V mutant RNA would be predicted to be cleaved in trans by pairing with the wild-type loop V of a separate RNA containing any loop I mutant.

Figure 9B,C shows that single mutants at position 631 in loop I or 698 in loop V cleave very slowly, if at all, in the spermine-containing buffer, as they did in our standard cleavage buffer (*18*). By ^{32}P-labeling one of the mutant RNAs and incubating with other unlabeled RNAs, we observed spermine-dependent cleavage when certain combinations of single mutant RNAs were mixed in the same test tube. For each of the three mutants in loop I that were examined (631G, A, or C) the best trans cleavage was obtained using an RNA containing the Watson−Crick complementary mutation at position 698 in loop V; the wobble pair 631G:698U was also quite active (Figure 9B).

Cleavage of each of the 698 mutants by any 631 mutant or by the wild-type was also observed, as expected for

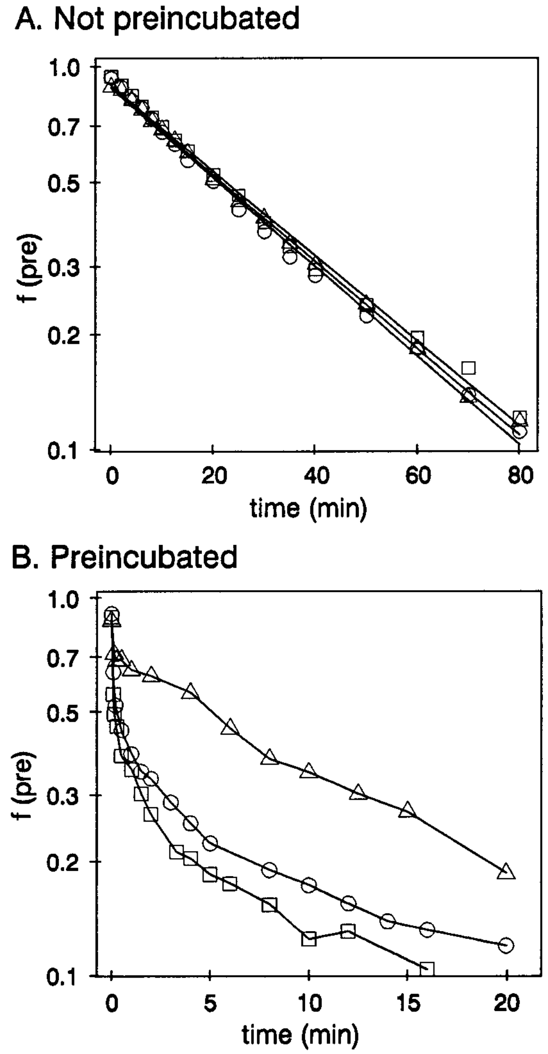


FIGURE 8: Effect of RNA concentration on the rate of the spermine-enhanced cleavage reaction. (A) RNA at final concentrations of 5 nM (triangles), 50 nM (circles), or 250 nM (squares) was preincubated for 30 min at 37 °C in 5 mM Tris-HCl pH 7.5, 5 μM EDTA in the absence of spermine; reactions were started by simultaneous addition of $MgCl_2$ to 2.5 mM and spermine to 100 μM. (B) As in (A) except that 100 μM spermine was included during the preincubation and the reactions were started by adding $MgCl_2$ to 2.5 mM.

recognition of the wild-type loop I in the 698 mutants by the wild-type loop V in the 631 mutants or the wild-type (Figure 9C). One loop I mutant, [^{32}P]631C, was analyzed further to confirm that cleavage by the complementary loop V mutant occurred only at a spermine concentration high enough to facilitate trans cleavage, i.e., at 100 μM but not 5 μM spermine (Figure 9D). At 5 μM spermine 631C was essentially inactive by itself or in the presence of wild-type or any 698 mutant RNA. At 100 μM spermine a small fraction of 631C RNA alone cleaved, suggesting that the higher spermine concentration may slightly stabilize this RNA; however, substantial cleavage was seen only in the presence of the complementary loop V mutant RNA, 698G. Spermine-dependent cleavage of the 698G/631C RNA combination also showed a fast burst followed by progressively slower cleavage, and the extent of the burst was dependent on RNA concentration (data not shown), as observed for wild-type G11 (see Figure 8). The sequence specificity of the cleavage of the 631 mutants by the

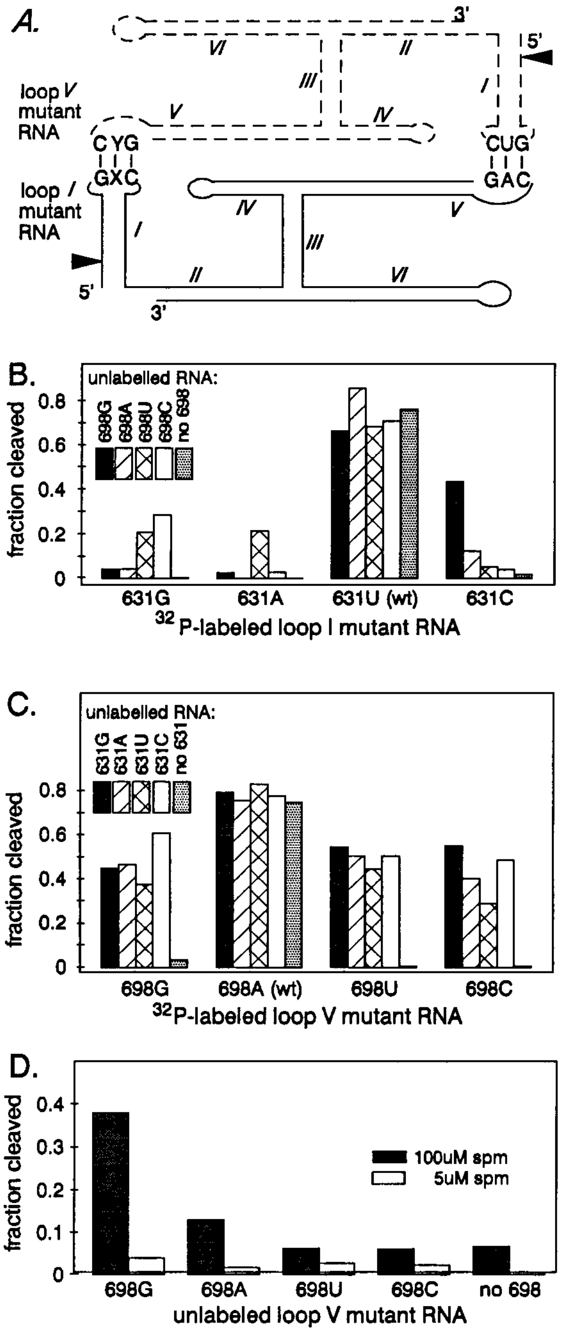


FIGURE 9: Spermine-dependent trans cleavage of mutants in the I/V pseudoknot. (A) Schematic diagram of the proposed intermolecular loop–loop interactions between two G11 Pre RNAs, one with a mutation at base 631 in loop I (indicated by X), the other with a mutation at its pairing partner, base 698 in loop V (indicated by Y). Helices are numbered by Roman numerals as in Figure 1. The cleavage site is indicated by the arrowhead. (B) One-point trans cleavage assays of ^{32}P-labeled wild-type and single-base substitution mutants at position 631 in loop I (indicated by X panel A) by unlabeled RNAs containing any of the four bases at position 698 in loop V (indicated by Y in panel A). Each combination of labeled and unlabeled RNAs (plus controls in which no unlabeled RNA was included) was preincubated for 30 min at 37 °C in 5 mM Tris-HCl pH 7.5, 5 μM EDTA in the presence of 100 μM spermine; reactions were started by addition of $MgCl_2$ to 2.5 mM and stopped after 30 s. Samples were resolved by electrophoresis, and the fraction of precursor cleaved was determined using a PhosphorImager. (C) as in (B), but the loop V mutant RNA was ^{32}P-labeled and the loop I mutant RNA was unlabeled. (D) Trans cleavage of [^{32}P]631C RNA by unlabeled 698G RNA at high but not low spermine concentration. Reactions were performed as described in (B), using 100 μM or 5 μM spermine.

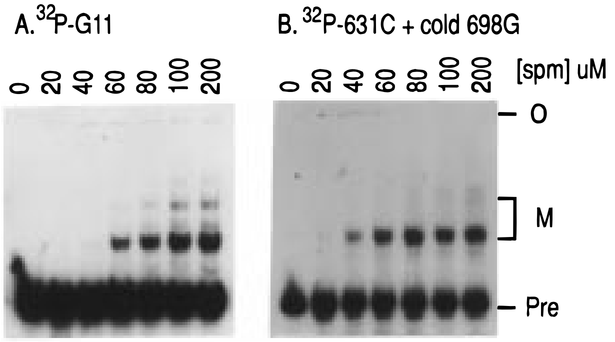


FIGURE 10: Spermine-dependent formation of multimeric RNAs. 250 nM [^{32}P]G11 RNA (A) or 125 nM [^{32}P]631C RNA plus 125 nM unlabeled 698G RNA (B) were preincubated in 5 mM Tris-HCl, pH 7.5, 5 μM EDTA for 30 min at 37 °C. One volume of loading solution (5 mM Tris-HCl, pH 7.5, 5 μM EDTA, 10% glycerol) was added, and RNAs were separated by electrophoresis on 4% polyacrylamide, 5 mM Tris-HCl, pH 7.5, 5 μM EDTA gels at room temperature with frequent buffer recirculation. Pre, monomeric precursor RNA; M, multimers; O, sample wells.

complementary 698 mutants confirms that cleavage occurs in trans and provides strong genetic evidence that the two (or more) RNAs interact via formation of an intermolecular version of the I/V pseudoknot.

To complement the kinetic and genetic data above, we also used nondenaturing gels to obtain physical evidence that spermine favored intermolecular RNA−RNA interactions. As done for the kinetic analyses, RNAs were preincubated in 5 mM Tris-HCl, pH 7.5, and 5 μM EDTA and a range of spermine concentrations, but instead of adding magnesium to start the cleavage reaction, samples were resolved by nondenaturing polyacrylamide gel electrophoresis (Figure 10). At low spermine concentrations, in which the kinetic data indicated no intermolecular interactions, the RNAs migrated as a single band. At higher spermine concentrations, which facilitated trans cleavage in kinetic experiments, a fraction of the RNA was found in bands that migrated substantially more slowly, as would be expected for multimers. The spermine-dependent formation of RNA multimers provides independent support for the idea that spermine induces intermolecular interactions.

Effects of Ionic Strength and pH on Spermine-Induced Trans Cleavage. Inclusion of even a few millimolar KCl, NaCl, or sodium acetate (NaAc) during preincubation of the RNA with spermine substantially decreased the extent of the burst of cleavage; above ∼10 mM salt, the burst was essentially prevented (Figure 11A). Even increasing the concentration of the buffer by an additional 10 to 20 mM prevented the burst of cleavage (Figure 11B). Similar effects of salt and buffer concentrations were observed using MES-KOH buffer pH 6.8 or Tris-HCl pH 7.5. These observations suggest that the structure of the spermine−RNA complex responsible for fast trans cleavage is extremely sensitive to ionic strength.

The rate and extent of the spermine-induced reaction were also affected by pH (Figure 11C,D). Above pH 6.5, the initial cleavage rate was too fast to measure accurately: using manual pipetting we could obtain only minimum estimates of the initial rates from time points removed 2−5 s into the reaction. Small differences in the extent of the burst were detectable between pH 6.5 and 8; above pH 8−8.5, the extent of the burst decreased markedly (Figure 11C). Below pH 6.5 the burst was not as evident, and the reaction rate decreased with decreasing pH (Figure 11D, and data not shown). Attempts to decrease the initial cleavage rate of the burst reactions into the measurable range by lowering the temperature of the reaction succeeded only in decreasing the fraction of the RNA that cleaved in the burst phase (Figure 11E).

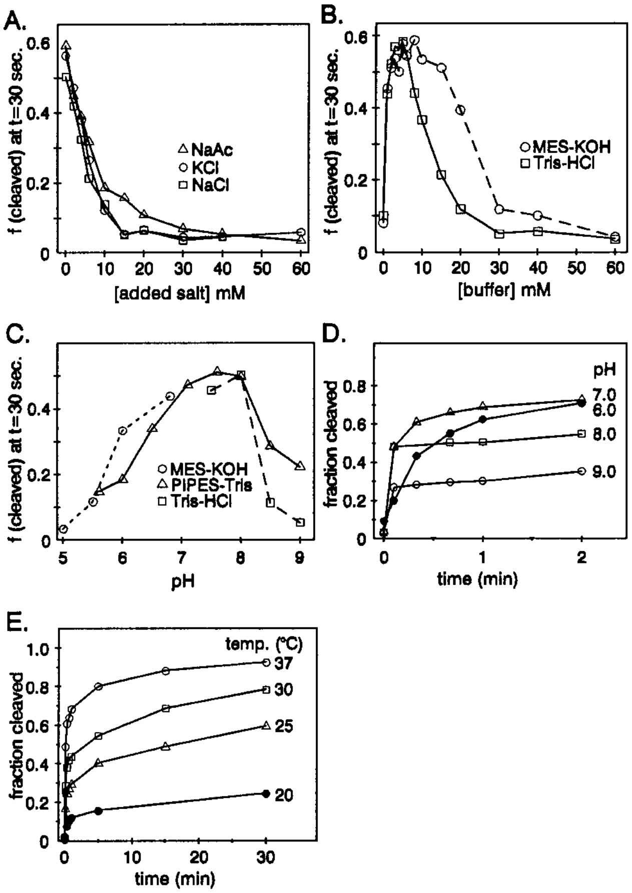


FIGURE 11: Effect of ionic strength, pH, and temperature on spermine-enhanced RNA cleavage. (A) RNA was preincubated for 30 min at 37 °C in 5 mM Tris-HCl pH 7.5, 5 μM EDTA, 100 μM spermine, and the indicated concentration of monovalent salts; reactions were started by addition of $MgCl_2$ to 2.5 mM and stopped after 30 s. (B) Preincubation and reactions were performed as in (A), except that the buffer concentration was varied as indicated on the *x*-axis. (C) Performed as in (A), except that the buffer composition and pH were varied as indicated on the *x*-axis. (D) Selected time courses of cleavage at different pH. Performed as in (A), except for pH 6.0, 7.0, and 9.0 in which the buffer was PIPES-Tris; aliquots were removed at the times indicated on the *x*-axis. (E) Performed as in (A), except that preincubations and reactions were at the temperatures indicated in the figure and aliquots were removed at the times indicated on the *x*-axis.

DISCUSSION

We describe both quantitative and qualitative effects of spermine on cleavage by the *Neurospora* VS ribozyme. When included in typical self-cleavage conditions spermine reduces the concentration of Mg^{2+} required for cleavage, as has also been observed with spermidine and with other ribozymes (*14*, *16*, *19*−*21*), and as is expected from the role of polyamines in stabilizing the secondary and tertiary structure of RNA (*11*, *12*, *22*). In contrast, when the RNA was preincubated with ≥20 μM spermine at low ionic strength, spermine caused a change in the nature of the cleavage reaction, switching the RNA from a slow cis-cleaving conformation into a conformation capable of rapid trans cleavage.

Analysis of the cleavage kinetics of wild-type RNA and of mutant RNAs that are incapable of cis cleavage suggests that spermine stabilizes a conformation of VS RNA in which the catalytic core is correctly folded but stem-loop I of a given RNA molecule, which contains the site of cleavage, does not dock into the core of the same molecule, perhaps because stem-loop I is stabilized in an alternative conformation, such as stacking on the end of helix II. In this model, loops I and V within a single RNA molecule could not interact in cis, but each would be otherwise properly structured and available for interactions with another RNA in trans (shown schematically in Figure 9A). Spermine may facilitate trans cleavage by stabilizing the alternative, trans-cleaving conformation. Because trans cleavage is observed at low RNA concentrations, even below 5 nM (Figure 8B), spermine also may act by bringing two or more molecules together, analogous to the role of polyamines in aggregation of DNA (*23, 24*).

Attempts to use chemical modification to probe the structure of VS RNA during preincubation with spermine have been unsuccessful because the low buffer concentration required during preincubation was not sufficient to maintain the pH against the byproducts of the chemical modification reactions (unpublished observations). As indirect evidence consistent with the above model, we have noticed that the downstream self-cleavage product of our standard construct, G11, in which the intramolecular loop I/V pseudoknot is quite stable (*25*), is not as efficient at trans cleavage as are ribozymes from which stem-loop I is completely removed (D. De Abreu and R. A. Collins, unpublished observations). This inefficiency in trans probably results from loop V being occupied by the cis loop I, thereby effectively reducing its opportunity to interact with a loop I on another RNA. In RNAs lacking stem-loop I, loop V is free to interact with a loop I on a separate RNA.

The spermine-induced trans cleavage is reminiscent of a similar effect of the basic, cyclic peptide antibiotic viomycin on cleavage of VS RNA. In a screen of antibiotics known to affect functions of other RNAs or RNA−protein complexes, we unexpectedly discovered that viomycin actually enhanced VS ribozyme cleavage by facilitating an intermolecular trans cleavage reaction somewhat similar to the effect of spermine described here (*26*). Preincubation of RNA and viomycin at low ionic strength was required for the RNA to adopt a conformation capable of trans cleavage. Some differences between the two enhancer molecules were also apparent: preincubation with viomycin worked best in water or low pH buffers (e.g., sodium succinate buffer pH 5.2; ref *26*, and unpublished data) while enhancement by spermine required at least a few millimolar buffer, probably to maintain the pH in the 7 to 8 range (Figure 11B,C). Also, the maximum rate of the spermine-enhanced reaction is at least 10-fold faster than the viomycin-enhanced reaction.

At ≥ 5 min^{-1}, the spermine-enhanced cleavage of G11 RNA is the fastest rate observed for any derivative of the VS ribozyme. Cis cleavage of G11 occurs at only ~ 0.08 min^{-1} (Figure 2A; ref *16*) and the pseudo-first-order rate of cleavage of stem-loop I RNA under single-turnover conditions by the trans-acting "Ava ribozyme" derivative of VS is ~ 0.7 min^{-1} (*8*). If the chemical cleavage step of the reaction involves hydroxide ions, it would be expected that the rate of the cleavage step would increase with increasing pH, as has been observed for some other hammerhead (*27*), RNase P (*28*−*30*) and Group I intron ribozymes (*31, 32*). However, we noted previously that the observed rates of cis- and trans-cleaving VS ribozymes were unaffected by pH (*8, 16*). In contrast, the spermine-enhanced reaction is affected by pH (Figure 11C,D), consistent with the possibility that the observed rate of the spermine-enhanced reaction is that of the chemical step. However, considering the complexity of the kinetics and the experimental system, the pH effect could alternatively reflect the protonation state of a functional group required for RNA−spermine interaction or one that facilitates a conformational change required for cleavage. Spermine-induced trans cleavage like that described here for VS RNA has not been reported for other ribozymes. It is possible that the trans cleavage phenomenon is unique to VS RNA; alternatively, it may not have been observed with other ribozymes because the very low ionic strength preincubation conditions required are not typically used in ribozyme studies.

ACKNOWLEDGMENT

We thank Angela Andersen, Tara Beattie, Diane De Abreu, Deborah Field, Toolika Rastogi, and Ricardo Zamel for helpful suggestions throughout the course of this work.

REFERENCES

1. Symons, R. H. (1992) *Annu. Rev. Biochem. 61,* 641−671.
2. Forster, A. C., Davies, C., Sheldon, C. C., Jeffries, A. C., and Symons, R. H. (1988) *Nature 334,* 265−267.
3. Uhlenbeck, O. C. (1987) *Nature 328,* 596−600.
4. Feldstein, P. A., Buzayan, J. M., van Tol, H., de Bear, J., Bough, G. R., Gilham, P. T., and Bruening, G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2623−2627.
5. Branch, A. D., and Robertson, H. D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 10163−10167.
6. Perrotta, A. T., and Been, M. D. (1992) *Biochemistry 31,* 16−21.
7. Wu, H.-N., Wang, Y.-J., Hung, C.-F., Lee, H.-J., and Lai, M. M. C. (1992) *J. Mol. Biol. 223,* 233−245.
8. Guo, H. C., and Collins, R. A. (1995) *EMBO J. 14,* 368−376.
9. Pan, T., Long, D., and Uhlenbeck, O. C. (1993) in *The RNA World* (Gesteland, R. F., and Atkins, J. F., Eds.) pp 271−302, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
10. Pyle, A. M., and Green, J. B. (1995) *Curr. Opin. Struct. Biol. 5,* 303−310.
11. Quigley, G. J. Teeter, M. M., and Rich, A. (1978) *Proc. Nat. Acad. Sci. U.S.A. 75,* 64−68.
12. Hanna, M., and Szostak, J. W. (1994) *Nucleic Acids Res. 22,* 5326−5331.
13. Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R., and Bruening, G. (1986) *Science 232,* 1577−1580.
14. Chowrira, B. M., Berzal-Herranz, A., and Burke, J. M. (1993) *Biochemistry 32,* 1088−1095.
15. Guo, H. C., De Abreu, D. M., Tillier, E. R., Saville, B. J., Olive, J. E., and Collins, R. A. (1993) *J. Mol. Biol. 232,* 351−361.
16. Collins, R. A., and Olive, J. E. (1993) *Biochemistry 32,* 2795−2799.

17. Daniels, D. L., Michels, W. J. J., and Pyle, A. M. (1996) *J. Mol. Biol. 256*, 31−49.
18. Rastogi, T., Beattie, T. L., Olive, J. E., and Collins, R. A. (1996) *EMBO J. 15*, 2820−2825.
19. Grosshans, C. A., and Cech, T. R. (1989) *Biochemistry 28*, 6888−6894.
20. Dahm, S. C., and Uhlenbeck, O. C. (1991) *Biochemistry 30*, 9464−9469.
21. Suh, Y.-A., Kumar, P. K. R., Taira, K., and Nishikawa, S. (1993) *Nucleic Acids Res. 21*, 3277−3280.
22. Tropp, J. S., and Redfield, A. G. (1983) *Nucleic Acids Res. 11*, 2121−2134.
23. Burgi, E., and Hershey, A. D. (1962) *J. Mol. Biol. 4*, 313−317.
24. Kaiser, D., Tabor, H., and Tabor, C. W. (1963) *J. Mol. Biol. 6*, 141−147.
25. Beattie, T. L., Olive, J. E., and Collins, R. A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4686−4690.
26. Olive, J. E., De Abreu, D. M., Rastogi, T., Andersen, A. A., Mittermaier, A. K., Beattie, T. L., and Collins, R. A. (1995) *EMBO J. 14*, 3247−3251.
27. Dahm, S. C., and Uhlenbeck, O. C. (1993) *Biochemistry 32*, 13040−13045.
28. Guerrier-Takada, C., Haydock, K., Allen, L., and Altman, S. (1986) *Biochemistry 25*, 1509−1515.
29. Smith, D., and Pace, N. R. (1993) *Biochemistry 32*, 5273−5281.
30. Beebe, J. A., and Fierke, C. A. (1994) *Biochemistry 33*, 10294−10304.
31. Herschlag, D., Eckstein, F., and Cech, T. R. (1993) *Biochemistry 32*, 8312−8321.
32. Herschlag, D., and Khosla, M. (1994) *Biochemistry 33*, 5291−5297.

BI972584W