Kinetics for Reaction of a Circularized Intervening Sequence with CU, UCU, CUCU, and CUCUCU: Mechanistic Implications from the Dependence on Temperature and on Oligomer and Mg^{2+} Concentrations†

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ABSTRACT: The self-splicing intervening sequence from the rRNA precursor in *Tetrahymena thermophila* produces a covalently closed, circularized form (C IVS). Reaction rates for reverse cyclization (linearization) of C IVS by the covalent addition of the oligonucleotides CU, UCU, CUCU, and CUCUCU have been measured. The dependence of the observed rates on oligomer and Mg^{2+} concentrations indicates the presence of intermediates that are generated by separate binding steps for both oligomer and Mg^{2+}. Linearization of C IVS by OH^- hydrolysis is suppressed in the presence of oligomer, suggesting oligomer binds near the active site. The binding constants derived for CU at 30 °C in 1 and 10 mM Mg^{2+} are 5×10^3 and 2.5×10^4 M^{-1}, respectively. These are roughly 4 orders of magnitude larger than expected for simple Watson–Crick base pairing. The binding constants derived for UCU, CUCU, and CUCUCU at 30 °C in 10 mM Mg^{2+} are 1.2×10^5, 4×10^5, and $>10^7$ M^{-1}, respectively. The free energy increments for binding of UCU and CUCU relative to CU are similar to those expected from a nearest-neighbor model for addition of base pairs. This indicates the factors responsible for the unusually strong binding of CU to C IVS are restricted to two nucleotides. The binding constant derived for Mg^{2+} in the presence of CU is 510 M^{-1} at 30 °C and is almost temperature independent. The temperature dependence is similar to that reported for other Mg^{2+}·RNA complexes. The binding constant, however, is smaller than most binding constants for Mg^{2+} to RNA, except for complexes involving inner-sphere complexation. The results suggest reverse cyclization of C IVS may involve binding of a weakly held Mg^{2+} ion. At 30 °C, rate constants for the reaction step involving transesterification with CU average roughly 0.05 and 0.4 h^{-1}, respectively, for cyclization and reverse cyclization. The rate for reverse cyclization is essentially independent of oligomer length. This indicates the rate of transesterification is not limited by the rate for disruption of the pairing between substrate and C IVS. The activation energy and entropy for reverse cyclization with CU are unusually large, 50 kcal/mol and 86 eu, respectively. The large, positive $\Delta S^{\ddagger}$ indicates the transition state for the rate-determining step is less ordered than the Mg^{2+}·C IVS·CU intermediate. This raises the possibility that a partial unfolding of the C IVS structure occurs during reaction. Thus, the "transesterification step" probably involves additional intermediates. The large activation energy indicates it should be possible to trap and study the Mg^{2+}·C IVS·CU intermediate.

It is known that RNA is able to catalyze the making and breaking of covalent bonds (Kruger et al., 1982; Zaug & Cech, 1986; Guerrier-Takada & Altman, 1984). The detailed mechanisms of these reactions are the subject of continued investigation. The first RNA known to facilitate cleavage and ligation reactions in RNA is the intervening sequence (IVS) from the rRNA precursor of *Tetrahymena thermophila* (Kruger et al., 1982; Cech & Bass, 1986). This RNA mediates a self-excision coupled to an exon splicing reaction and subsequently undergoes a self-circularization reaction (Zaug et al., 1983). The circular product (C IVS) can be linearized by addition of oligoribonucleotides, and this new product is itself able to undergo circularization in a reversible manner (Sullivan & Cech, 1985). These last reactions provide a convenient model system for studying the mechanism of IVS reactions. Since all the reactions are transesterifications, it is likely the mechanisms are similar.

In this paper, we report the kinetics in the minutes to hours time range for the linearization of C IVS with the oligoribonucleotides CU, UCU, CUCU, and CUCUCU. The kinetics as a function of oligomer and Mg^{2+} concentration and of temperature suggest the mechanism involves separate binding steps for both oligomer and Mg^{2+}. The binding constants deduced for the oligomers are anomalously large. The anomalous component of the binding is apparently restricted to two nucleotides, however. The binding constant deduced for Mg^{2+} is small, but consistent with formation of an inner-sphere complex. The activation energy and entropy for reverse cyclization are unusually large and raise the possibility that a conformational change is required to form the transition state.

Materials and Methods

Nucleic Acids. CU was obtained from Sigma, and purity was confirmed by high-performance liquid chromatography. UCU, CUCU, and CUCUCU were synthesized on solid support with a phosphoramidite method (Kierzek et al., 1986) and purified by high-performance liquid chromatography (Ikuta et al., 1984). Concentrations were determined optically with extinction coefficients of 1.67×10^4, 2.66×10^4, $3.33 \times$

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10^4, and 4.98×10^4 M^{-1} cm^{-1} at 260 nm for CU, UCU, CUCU, and CUCUCU, respectively (Richards, 1975).

^{32}P-Labeled C IVS was obtained by transcribing the plasmid pTT1A3-T7 (Zaug et al., 1986; Zaug & Cech, 1986) with T7 RNA polymerase (Davanloo et al., 1984) in the presence of [α-^{32}P]ATP. The transcription buffer was 40 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.5), 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, and 1 mM NTPs. pTT1A3-T7 (5 μg) digested with *Eco*RI and 10 units/μL T7 polymerase were incubated in 50 μL of reaction mixture for 1 h at 37 °C. The reaction mixture was added to 2.5 μL of 4 M NaCl and 150 μL of splicing buffer [5 mM $MgCl_2$, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 0.24 M NaCl, and 0.1 mM GTP]. The mixture was incubated for 30 min at 37 °C to promote excision and cyclization of the IVS. The RNA was separated on a 4% polyacrylamide/8 M urea gel, and C IVS was visualized by ethidium bromide staining, cut out, eluted, and purified by chromatography on Sephadex G-50. Unlabeled C IVS was obtained in a similar manner and its concentration determined optically with an extinction coefficient of 3.2×10^6 M^{-1} cm^{-1} at 260 nm (A. J. Zaug, C. Grosshans, and T. R. Cech, personal communication).

Reverse Cyclization Reactions. The buffer for reverse cyclization reactions was 10 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 6.5, containing various concentrations of $MgCl_2$. For calculations and plotting of results, it was assumed that 0.5 mM Mg^{2+} was complexed with EDTA. For example, a solution containing a total Mg^{2+} concentration of 10.5 mM was assumed to have a free Mg^{2+} concentration of 10 mM. The buffer was chosen to minimize random and specific (Zaug et al., 1984) OH^- hydrolysis of C IVS. To ensure pseudo-first-order conditions, the concentration of C IVS was on the order of 10^{-8} M, always much less than the concentrations of CU and Mg^{2+}.

Reactions were run in 20-μL volumes sealed in glass capillaries. Constant temperature was ensured by incubating the capillaries in a Lauda RC6 bath. Reactions were stopped by plunging into ice water and subsequently adding EDTA to a final concentration of 25 mM.

Reaction mixtures were analyzed by running on denaturing 4% polyacrylamide gels, cutting out bands corresponding to circular and linear species, and quantifying radioactivity by scintillation counting (Sullivan & Cech, 1985).

Data Analysis. The extent of reaction was defined as [CU-L′IVS]/([CU-L′IVS] + [C IVS]). Here CU-L′IVS represents C IVS that has been linearized by covalent addition of oligomer at A16. The extent of reaction vs time points was analyzed with the computer program DISCRETE written by Provencher (1976a,b). The program was allowed to fit up to two exponentials for each time course. In all cases below 40 °C, the single-exponential fit was excellent.

Nonlinear least-squares fits of observed rate constants to kinetic equations were done with a program written by L. Friedrich (Eastman Kodak Co.). Error bars were included in the least-squares algorithm.

RESULTS

Results for CU at 30 °C. Typical examples of the time courses of the reaction of C IVS with CU at 30 °C in 10.5 mM $MgCl_2$, 0.5 mM EDTA, 10 mM NaCl, and 10 mM PIPES, pH 6.5, are shown in Figure 1. Also shown are the results from a typical control experiment in which CU was omitted. In the absence of CU, the circle is opened specifically at G414–A16 by reaction with OH^- (Zaug et al., 1984, 1985).

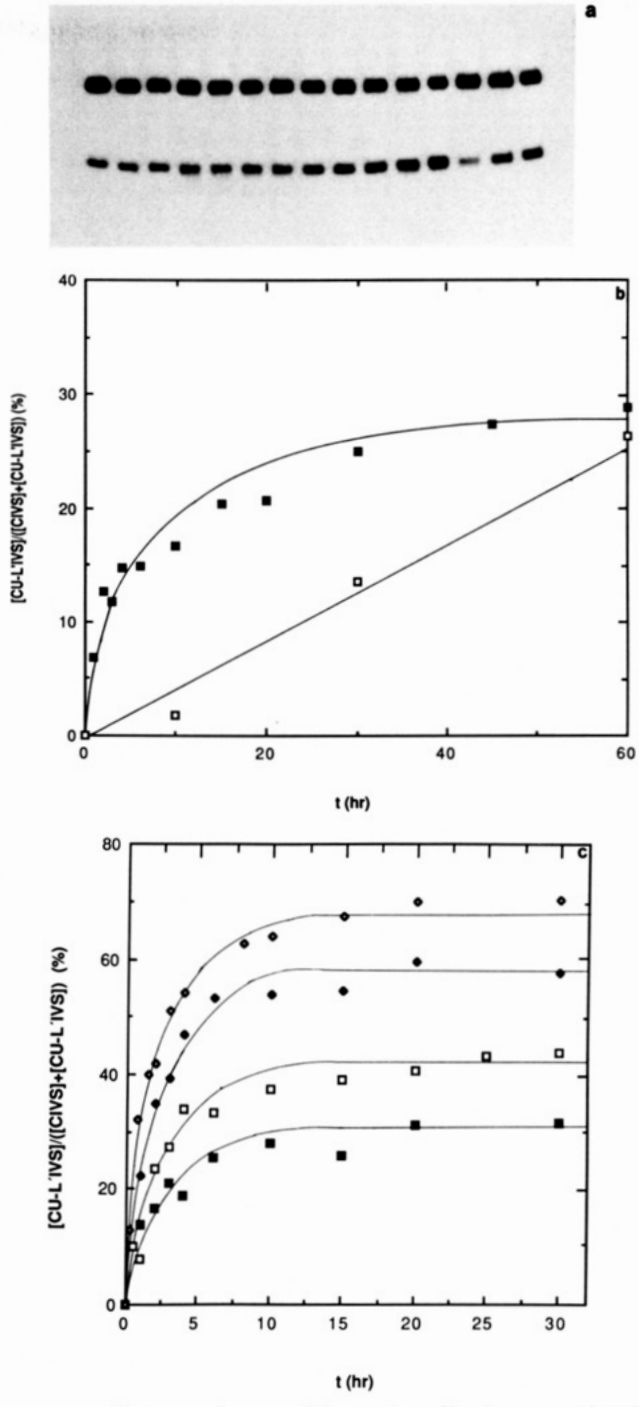

FIGURE 1: (a) Autoradiogram of denaturing 4% polyacrylamide gel showing time dependence of reverse cyclization by CU. Circular IVS was incubated at 30 °C with 0.01 mM CU in 10 mM NaCl, 10.5 mM $MgCl_2$, 0.5 mM EDTA, and 10 mM PIPES, pH 6.5. From left to right, incubation times are 0, 1, 2, 3, 4, 6, 10, 15, 20, 30, 45, and 60 h. Reactions shown in the last three lanes were run in the absence of CU with incubation times (left to right) of 10, 30, and 60 h. (b) Extent of reverse cyclization vs time in the absence of CU (□) and in the presence of 0.01 mM CU (■). Points were corrected for linear species present at $t = 0$, so that the extent of reaction is 0 at $t = 0$ for each concentration. Buffer was 10 mM NaCl, 10.5 mM $MgCl_2$, 0.5 mM EDTA, and 10 mM PIPES, pH 6.5. Solid curve is the nonlinear least-squares fit of data points to the single-exponential function of eq 1. (c) Extent of reverse cyclization at 30 °C vs time for the following concentrations of CU: (■) 0.05 mM, (□) 0.1 mM, (◆) 0.5 mM, and (◇) 1.0 mM. Same conditions as for (b).

As shown previously (Zaug et al., 1984; Sullivan & Cech, 1985), circle opening by OH^- is much slower than by oligonucleotide. The time courses in the absence and presence of CU indicate CU inhibits circle opening by OH^-. This is shown

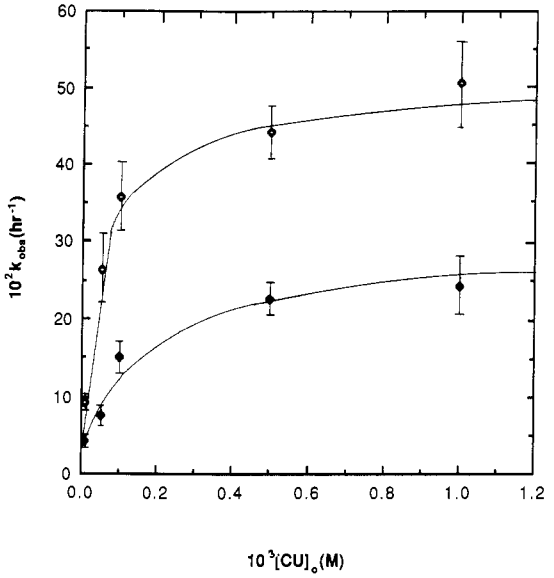

FIGURE 2: Plot of k_{obsd} at 30 °C vs concentration of CU in the presence of (◇) 10 mM free Mg^{2+} and (◆) 1 mM free Mg^{2+}. Buffer was 10 mM NaCl, 0.5 mM EDTA, and 10 mM PIPES, pH 6.5. Solid lines are the nonlinear least-squares fits to eq 4.

by the change in extent of reaction at long times. From roughly 15 to 60 h, the change in CU-L'IVS is minimal when [CU] ≥ 0.05 mM, but is significant in the absence of CU. The extent of circle opening by OH^- was also tested by incubating ~10^{-8} M CU labeled with ^{32}P on the 5'-end with 8 × 10^{-6} M unlabeled C IVS for 120 h under the conditions listed in the legend to Figure 1. The extent of reaction was determined by running the reaction mixture and an aliquot containing an equal amount of labeled CU on separate 4% polyacrylamide gels and cutting out and counting the CU and CU-L'IVS bands. Twenty-four percent of the labeled CU reacted to give labeled CU-L'IVS. This compares well with the 27% extent of reaction measured with 10^{-5} M unlabeled CU and ~10^{-8} M labeled C IVS (see Figure 1b). This agreement indicates essentially all the cricle opening is due to reaction with CU. Thus, it was assumed that circle opening by OH^- is negligible in the presence of CU, and therefore, no correction for OH^- hydrolysis was added before analysis of the data at 30 °C.

The time dependence of the fraction of the linear form [CU-L'IVS]/([CU-L'IVS] + [C IVS]) is represented well by a single exponential:

$$\frac{[\text{CU-L'IVS}]}{[\text{CU-L'IVS}] + [\text{C IVS}]} = \left[\frac{[\text{CU-L'IVS}]}{[\text{CU-L'IVS}] + [\text{C IVS}]}\right]_{t=\infty} (1 - e^{-k_{obsd}t}) \quad (1)$$

Curves fit to eq 1 by the method of Provencher (1976a,b) are also shown in Figure 1. The final extent of reaction increases with increasing CU concentration as expected for an equilibrium reaction. At high CU concentrations, however, the final extent of reaction is not as large as expected. This may indicate that some of the C IVS is not reactive. A similar effect was observed by Sullivan and Cech (1985). Since CU is in large excess, this should not affect the kinetic results. Plots of the observed first-order rate constant k_{obsd} as a function of the initial concentration of CU, $[CU]_0$, are shown in Figure 2 for both 1 and 10 mM Mg^{2+}. $[CU]_0$ ranged from 10^{-5} to 10^{-3} M.

In both 1 and 10 mM Mg^{2+}, k_{obsd} is essentially maximized at 1 mM CU (see Figure 2). The maximum rates, however, differ by a factor of ~2. Therefore, k_{obsd} at 1 mM CU was

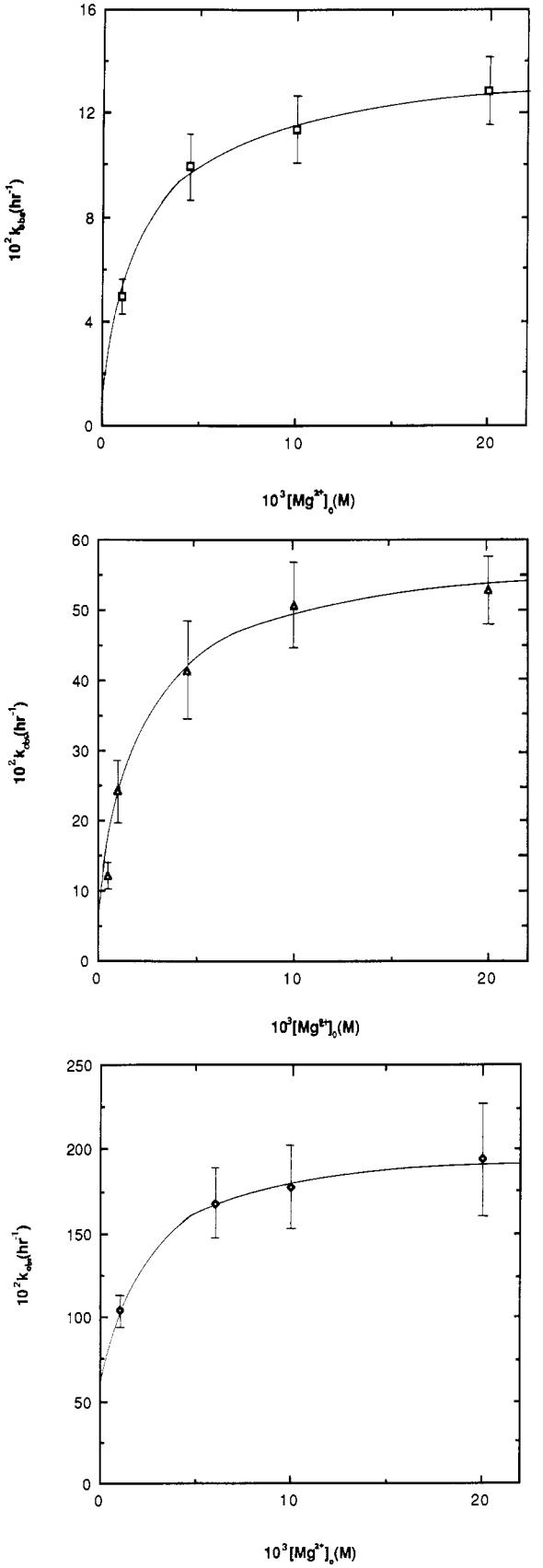

FIGURE 3: Plots of k_{obsd} at 25 (□), 30 (△), and 35 °C (◇) in the presence of 1 mM CU vs concentration of free Mg^{2+}. Buffer was 10 mM NaCl, 0.5 mM EDTA, and 10 mM PIPES, pH 6.5. Solid lines are the nonlinear least-squares fits to eq 6.

measured for free Mg^{2+} concentrations ranging from 0.5 to 20 mM. The results are shown in Figure 3. Evidently, the maximal k_{obsd} is reached by 10 mM Mg^{2+}, and k_{obsd} decreases

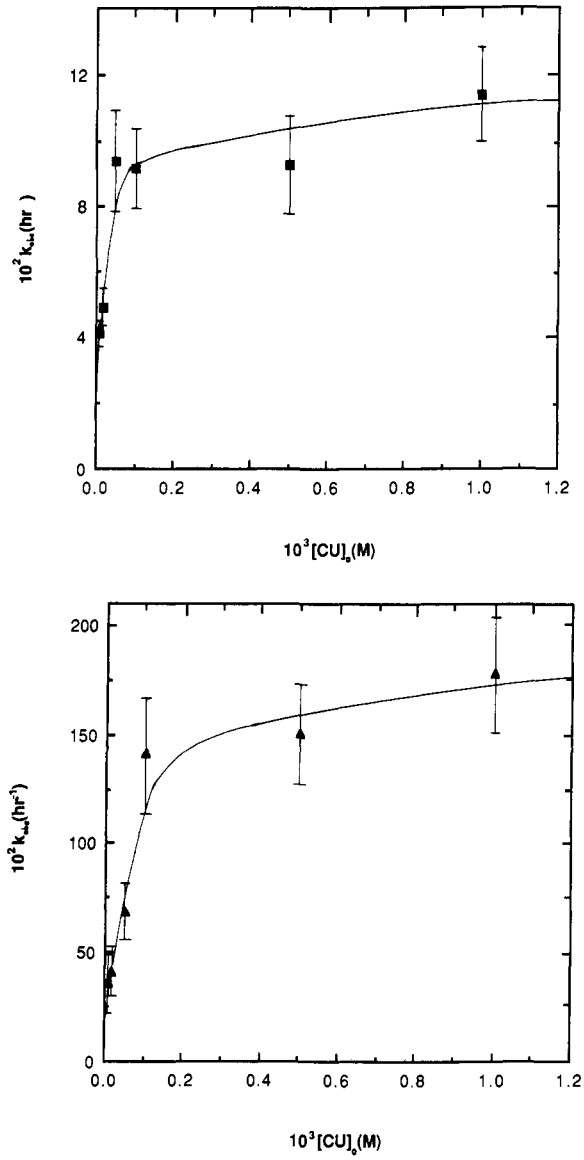

FIGURE 4: Plots of k_{obsd} at 25 (■) and 35 °C (▲) vs concentration of CU in 10 mM free Mg^{2+}, 10 mM NaCl, 0.5 mM EDTA, and 10 mM PIPES, pH 6.5. Solid lines are the nonlinear least-squares fits to eq 4.

hyperbolically at lower concentrations.

The effect of increasing ionic strength was determined by measuring k_{obsd} at 1 mM CU in 100 mM NaCl/10 mM free Mg^{2+}. The kinetic curve is given in the supplementary material (see paragraph at end of paper regarding supplementary material), and k_{obsd} is 0.23 h^{-1}. This is roughly 2 times smaller than the rate constant of 0.51 h^{-1} observed in 10 mM NaCl/10 mM free Mg^{2+} (see Figure 2). Within experimental error, it is the same as the rate constant of 0.24 h^{-1} observed in 10 mM NaCl/1 mM free Mg^{2+} (see supplementary material). Thus, under these conditions, increasing the NaCl concentration a factor of 10 slows the reaction by the same factor as decreasing the $MgCl_2$ concentration a factor of 10.

Temperature Dependence. The kinetics of reverse cyclization was also studied at different temperatures. At 25 and 35 °C, the time dependence of the fraction of linear form was represented well by a single exponential. Typical plots are shown in the supplementary material. The dependences of k_{obsd} on CU and Mg^{2+} concentration at 25 and 35 °C are shown in Figures 3 and 4.

At 42 and 50 °C, the time dependence of the fraction of linear form was not a single exponential. Typical results are

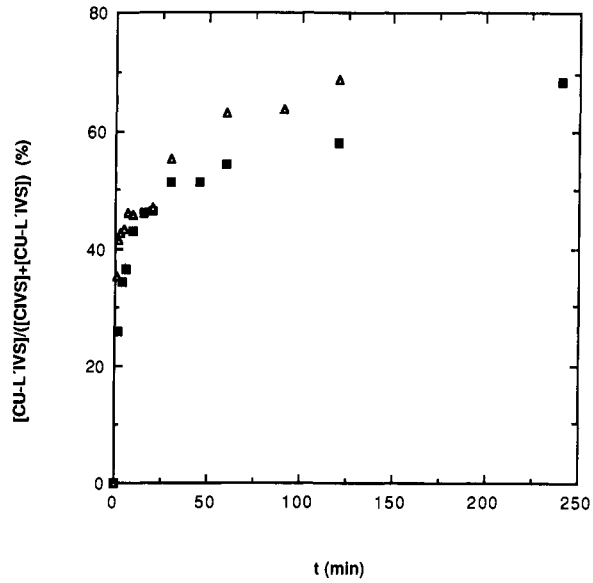

FIGURE 5: Extent of reverse cyclization vs time at 42 (■) and 50 °C (△) for 1 mM CU in 10 mM NaCl, 10.5 mM $MgCl_2$, 0.5 mM EDTA, and 10 mM PIPES, pH 6.5. When the data is fit to a double exponential, the k_{obsd} are 23 and 0.5 h^{-1} at 42 °C and 100 and 1 h^{-1} at 50 °C. If the curves are truncated at 120 and 20 min, respectively, for 42 and 50 °C, the k_{obsd} are 13 and 60 h^{-1}.

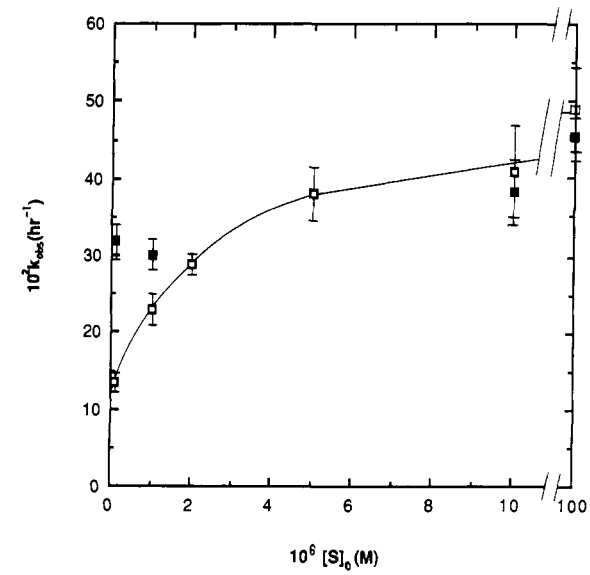

FIGURE 6: Plots of k_{obsd} at 30 °C vs concentrations of (□) CUCU or (■) CUCUCU. Buffer was 10 mM NaCl, 10.5 mM $MgCl_2$, 0.5 mM EDTA, and 10 mM PIPES, pH 6.5. Solid line is the nonlinear least-squares fit to eq 4.

shown in Figure 5, and the kinetic parameters are listed in the figure caption.

Sequence Dependence. Rates for reverse cyclization were also measured for UCU, CUCU, and CUCUCU at 30 °C with 10 mM free Mg^{2+}. Typical results are shown in Figure 6 and in the supplementary material.

Discussion

The circularized form of the self-splicing intervening sequence can be opened specifically at the G414–A16 phosphodiester linkage by either hydrolysis with OH^- (Zaug et al., 1984) or attack by oligoribonucleotides (Sullivan & Cech, 1985). For the conditions used in the experiments reported here, the circle is opened much slower by reaction with OH^- than with CU (see Figure 1). In the presence of CU, reaction with OH^- is even slower than in the absence of CU. Thus,

Table I: Kinetic Constants for Circle Opening by CU or UUU

oligomer	ref and buffer	free [Mg^{2+}] (mM)	T (°C)	$K_1 \times 10^{-3}$ (M^{-1})	k_2 (h^{-1})	k_{-2} (h^{-1})
CU	*a*	1	30	5 ± 2	0.27 ± 0.05	0.03 ± 0.01
CU	*a*	10	25	41 ± 20	0.10 ± 0.03	0.01 ± 0.03
		10	30	25 ± 4	0.50 ± 0.03	*c*
		10	35	11 ± 4	1.8 ± 0.3	0.1 ± 0.2
UCU	*a*	10	30	120 ± 20	0.37 ± 0.01	*c*
CUCU	*a*	10	30	400 ± 20	0.39 ± 0.01	0.12 ± 0.01
CUCUCU	*a*	10	30	>10000[d]	(0.3)[d]	
UUU	*b*	10	42	19	24	2

oligomer	ref and buffer	[CU] (mM)	T (°C)	K_a (M^{-1})	k_b (h^{-1})	k_{-b} (h^{-1})
CU	*a*	1	25	500 ± 220	0.13 ± 0.01	0.01 ± 0.02
		1	30	510 ± 260	0.51 ± 0.06	0.07 ± 0.02
		1	35	420 ± 150	1.5 ± 0.1	0.6 ± 0.3

[a] This work. Buffer is 10 mM NaCl, 0.5 mM EDTA, 10 mM PIPES, pH 6.5, and indicated concentration of $MgCl_2$ or varying concentrations of $MgCl_2$. [b] Sullivan and Cech (1985). Buffer is 100 mM $(NH_4)_2SO_4$/30 mM Tris, pH 7.5. [c] Could not be determined. [d] For CUCUCU, k_{obsd} changes little between 10^{-7} and 10^{-4} M, suggesting $K_1 > 10^7$ M. Lower concentrations were not studied because the derivation of eq 4 requires the concentration of oligomer to be much larger than the concentration of C IVS. The estimate of k_2 was obtained from the limiting k_{obsd} by assuming the same k_{-2} as for CUCU.

the kinetic results reported here for CU between 25 and 35 °C relate only to the overall reaction

$$\text{C IVS} + \text{CU} \rightleftarrows \text{CU-L'IVS} \quad (2)$$

The Results Require a Noncovalent Intermediate. If the mechanism for circle opening by CU was the same as eq 2, then for [CU] ≫ [C IVS], the observed rate constant would increase linearly with CU concentration. As shown in Figure 2, this is not observed.

The simplest mechanism consistent with the results in Figure 2, is

$$\text{C IVS} + \text{CU} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{C IVS·CU} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{CU-L'IVS} \quad (3)$$

Here C IVS·CU is a noncovalent complex of circle with CU. When the first step in the mechanism is much faster than the second step and [CU] ≫ [C IVS], the observed rate constant for this mechanism is (see Appendix)

$$k_{obsd} = \{k_2[CU]_0/([CU]_0 + 1/K_1)\} + k_{-2} \quad (4)$$

Here $[CU]_0$ is the initial concentration of CU and $K_1 = k_1/k_{-1}$. Nonlinear least-squares fits to eq 4 of the data shown in Figures 2 and 4 provide values for k_2, k_{-2}, and K_1. These are listed in Table I, and the fitted curves are shown in Figures 2 and 4. Equation 4 also fits the data for UCU, CUCU, and CUCUCU, and of Sullivan and Cech (1985) for UUU at 42 °C in 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, and 30 mM Tris, pH 7.5. The constants derived for reaction with these other oligomers are also listed in Table I. The values of K_1 and k_2 for UUU are similar to those derived from the same data by Cech and Bass (1986) using a Michaelis–Menten treatment. This is reasonable since eq 4 reduces to the Michaelis–Menten equation when k_{-2} is zero.

The equilibrium constant for the second reaction step, $K_2 = k_2/k_{-2}$, is ~10. Thus, the second step in the mechanism is associated with a relatively small change in free energy, $\Delta G°_2 = -RT \ln K_2 \approx -1.4$ kcal/mol. This is consistent with the second step corresponding to the process of transesterification. Since transesterification involves the breakage and subsequent formation of a phosphodiester bond, it is expected to provide a relatively small change in free energy.

Binding of CU in the Intermediate Is Unusually Strong. Presumably, the first step in the mechanism involves binding of CU to circle. The inhibition by CU of circle opening by OH^- is consistent with CU being bound close to the G414–A16 phosphodiester bond. Similar inhibition has been observed previously with deoxyguanosine and dideoxyguanosine bound to rRNA precursor and attributed to similar steric effects (Bass & Cech, 1986). The values of K_1 deduced for the binding of CU to C IVS at 30 °C are 5000 and 25000 M^{-1}, respectively, in 1 and 10 mM Mg^{2+}. These are large compared with binding constants expected for CU binding by simple base pairing. For example, on the basis of phylogenetic data (Davies et al., 1982; Waring et al., 1983), site-directed mutagenesis (Been & Cech, 1987), and the sequence dependence of circle opening (Sullivan & Cech, 1985; Been & Cech, 1987), it is reasonable to assume that CU is bound to part of the internal guide sequence: $^{3'UC5'}_{5'GGR3'}$. Here R is a purine. The binding constant for this association can be predicted from nearest-neighbor parameters determined for RNA duplex formation (Freier et al., 1986). The predicted value is ~0.1 M^{-1} in 1 M NaCl at 30 °C if only base pairs are considered. If the effect of the dangling 3′-R is added (Freier et al., 1983; Sugimoto et al., 1987), the predicted value is 1 M^{-1}. Experiments on the melting temperatures of the deoxyoligonucleotide duplexes $(dG\text{-}dT)_5\cdot(dA\text{-}dC)_5$ and dGCATGC suggest the predicted value will be very similar for the salt conditions used here (A. Williams and D. H. Turner, unpublished results). The difference between the measured K_1's and the predicted binding constant is equivalent to an additional 5–6 kcal/mol in binding free energy. Evidently, the first step in the reaction involves more than a simple association of CU to circle by base pairing.

Previous results have also indicated the structure of the self-splicing intervening sequence is able to bind oligonucleotides with unusually large binding constants. For example, Zaug and Cech (1986) have studied the inhibition by dC_5 of a polymerization reaction catalyzed by L-19 IVS and deduced a binding constant of 3800 M^{-1} for dC_5. The Michaelis constant for the rC_5 substrate in this reaction suggests a binding constant of 24000 M^{-1} at 42 °C in 20 mM Mg^{2+}. As shown in Table I, the data of Sullivan and Cech (1985) for circle opening by UUU at 42 °C is best fit to eq 4 with a K_1 of 19000 M^{-1}.

Lengthening the Substrate Does Not Increase Binding More Than Expected for Base Pairing. If the unusual binding interactions measured with CU were duplicated in both CUs of CUCU, then the binding constant for CUCU would be larger than 5×10^8 M^{-1}. The measured apparent binding constant for CUCU is only 4×10^5 M^{-1} (see Table I). Thus, the extent of these special interactions is restricted.

Free energy increments for substrate binding as a function of substrate length can be compared with increments expected for base pair formation to provide an indication of the extent of special interactions. The expected increments can be pre-

Table II: Measured and Predicted Free Energy Increments Relative to CU for Binding of Oligomers to Circle

	measured		predicted	
oligomer	$\Delta G°_{30}$ (kcal/mol)	$\Delta\Delta G°_{30}$ (kcal/mol)	$\Delta\Delta G°_{30}$ 5′GGAG3′ site (kcal/mol)	$\Delta\Delta G°_{30}$ 5′GGGA3′ site (kcal/mol)
CU	−6.1			
UCU	−7.0	−0.9	−2.2	−1.3
CUCU	−7.8	−1.7	−4.4	−1.5
CUCUCU	<−10	<−4	−7	*a*

[a] The parameters required for this prediction have not been measured.

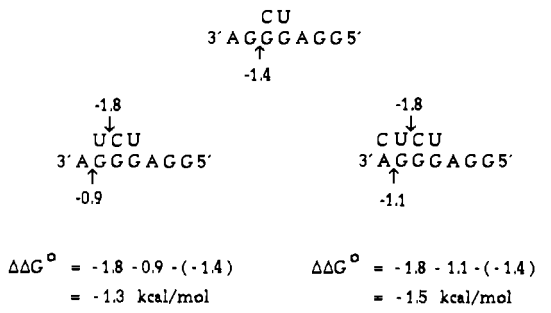

FIGURE 7: Calculation of free energy increments at 30 °C for binding of UCU and CUCU relative to CU. Free energy increments are in kilocalories per mole (Freier et al., 1986; Turner et al., 1988). A terminal GU pair is treated as an AU pair (Freier et al., 1986).

dicted from the nearest-neighbor model for duplex formation by oligoribonucleotides (Borer et al., 1974; Kierzek et al., 1986; Freier et al., 1986). The substrate free energy increments can be obtained by subtracting $\Delta G°_{30}$ for binding of CU from $\Delta G°_{30}$ for binding of the longer oligomers. These increments are listed in Table II. Comparison with predicted increments requires knowledge of the binding site. It was originally thought that the binding site for reverse cyclization was the "internal guide sequence" 5′GGAGGGA3′ used by the 3′-end of the 5′-exon 5′CUCUCU3′ during splicing (Davies et al., 1982; Waring et al., 1983; Sullivan & Cech, 1985). Recent results from site-directed mutagenesis, however, suggest reverse cyclization involves only the GGGA part of the internal guide sequence (Been & Cech, 1987). Preliminary nuclease footprinting experiments in the presence of the deoxyoligonucleotide inhibitor dCTCT are in agreement with the latter proposal (N. Sugimoto and D. H. Turner, unpublished results). The binding free energy increments relative to CU predicted from the nearest-neighbor model for each oligomer studied are listed in Table II for both potential binding sites. Figure 7 illustrates the calculations for UCU and CUCU binding to GGGA. The predicted increments relative to CU are −1.3 and −1.5 kcal/mol for this interaction. They are in reasonable agreement with the measured values of −0.9 and −1.7 kcal/mol. The results suggest the nearest-neighbor model will be useful for predicting free energy increments for lengthening substrates and/or binding sites. It also suggests the unusual nature of substrate binding to C IVS is restricted to the first one or two nucleotides of the 3′-end of the substrate.

Increasing the length of oligomer substrate clearly increases the binding constant between substrate and C IVS. Since the association rates of oligonucleotides have little dependence on length (Porschke et al., 1973; Turner et al., 1989), this suggests the dissociation rate of substrate from C IVS is quite length dependent. Substrate length has little or no effect on k_2, however. This is the rate constant assigned to the step involving transesterification. This result indicates the rate of transesterification is not limited by the rate for disruption of the pairing between substrate and internal guide sequence.

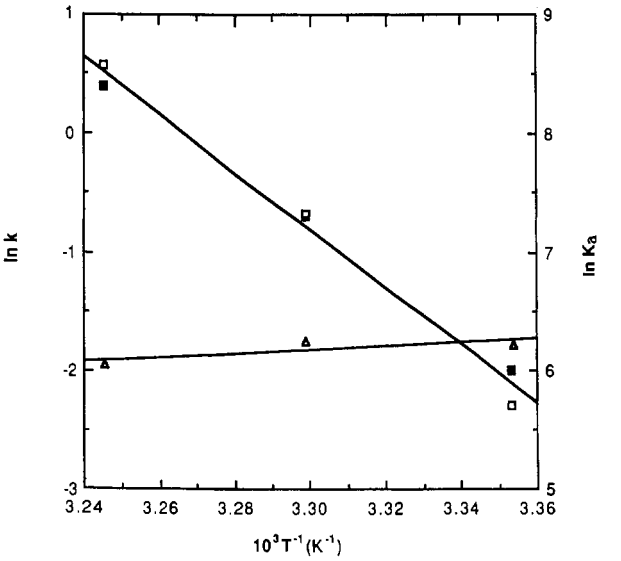

FIGURE 8: Arrhenius plot of ln k_2 (□) and ln k_b (■) vs $1/T$ and van't Hoff plot of ln K_a (△) vs $1/T$. The solid lines are the linear least-squares fits to the data. The derived values for E_a and ΔS^* are 50 kcal/mol and 86 eu, respectively. The derived values for ΔH_a and ΔS_a are −3 kcal/mol and 2 eu, respectively.

The Mechanism May Also Involve Uptake of a Mg^{2+} Ion. At 30 °C, in both 1 and 10 mM Mg^{2+}, the observed rate constant for circle opening is essentially saturated at 1 mM CU. Nevertheless, the rates observed in 1 and 10 mM Mg^{2+} differ by a factor of 2. The results in Figure 3 indicate that, in 10 mM Na^+, the dependence of k_{obsd} on $[Mg^{2+}]$ is saturated by 10 mM Mg^{2+} and falls off slowly between 10 and 1 mM Mg^{2+}. One simple mechanism consistent with this dependence involves uptake of a Mg^{2+} ion during the course of the reaction, e.g.:

$$\text{C IVS·CU} + Mg^{2+} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} Mg^{2+}\text{·C IVS·CU} \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} \text{CU-L′IVS·}Mg^{2+} \quad (5)$$

Alternatively, Mg^{2+} could be taken up by C IVS before CU is bound. The mechanism of eq 5 is analogous to eq 3. When $[Mg^{2+}] \gg$ [C IVS], the equation for the observed rate constant is analogous to eq 4:

$$k_{obsd} = \{k_b[Mg^{2+}]_0/([Mg^{2+}]_0 + 1/K_a)\} + k_{-b} \quad (6)$$

Here $[Mg^{2+}]_0$ is the initial concentration of free Mg^{2+}, and $K_a = k_a/k_{-a}$. Nonlinear least-squares fits of the data at 25, 30, and 35 °C give values for K_a, k_b, and k_{-b}. These are listed in Table I, and the fitted curves are shown in Figure 3. Comparison of eq 5 and 3 suggests the second steps in both mechanisms are the same. The similarities between the derived values of k_2 and k_b and between k_{-2} and k_{-b} are consistent with this suggestion. The ratios k_2/k_{-2} and k_b/k_{-b} apparently have opposite dependences on temperature, however. This is likely due to the difficulty in determining k_{-2} and k_{-b} rather than a real difference. Thus, the second step in mechanism 5 probably involves transesterification.

The Temperature Dependence Is Unusual and Suggests Partial Unfolding of Structure. The temperature dependence of k_2 and k_b provides activation parameters for the step involving transesterification. An Arrhenius plot of the data is shown in Figure 8. Assuming k_2 and k_b are for the same process, the activation energy, E_a, is 50 kcal/mol. The activation entropy, ΔS^*, is derived from the Eyring equation:

$$k = \frac{eRT}{Nh} \exp(-E_a/RT) \exp(\Delta S^*/R) \quad (7)$$

Table III: Literature Values for Binding Constants of Mg^{2+} to RNA

nucleic acid	ref	type of binding	pH	buffer[a]	T (°C)	K	ΔH° (kcal/mol)	ΔS° (eu)
$A(pA)_4$	b	inner sphere	5.9	1 mM Na Caco	20	3.4×10^3		
$A(pA)_{17}$	b	outer sphere "ion atmosphere"	8	0.5 mM Tris-HCl	20	1.9×10^5		
anticodon loop of yeast phe tRNA	c	inner sphere	7.1	100 mM $NaClO_4$ 50 mM Tris–Caco	30	1.2×10^3	−3.9	1
yeast Phe-tRNA	d	weak	7.2	5 mM NaCl 5 mM sodium phosphate	25	1.1×10^4	0	18.4
poly(A)·poly(U)	e,f	outer sphere "ion atmosphere"		10 mM Na^+	20	7.9×10^4	2	
				100 mM Na^+	20	4.0×10^2		

[a] Caco is cacodylate. [b] Pörschke (1979). [c] Bujalowski et al. (1986). [d] Rialdi et al. (1972). [e] Archer et al. (1972). [f] Krakauer (1971).

Here e is the base of natural logarithms (2.72), N is Avogadro's number, and h is Planck's constant. The activation entropy is 86 eu. Both the activation energy and entropy are unusually large. The large positive activation entropy is particularly novel. The positive sign implies the transition state for transesterification is less ordered than the Mg^{2+}·C IVS·CU intermediate. One possible origin for such disorder is partial unfolding of some part of the C IVS structure. For example, thermodynamic parameters for base pairing (Freier et al., 1986) indicate breaking one of the conserved helixes P3, P4, or P7 (Waring et al., 1983, 1986) would be associated with entropy changes on the order of 100 eu. An alternative possibility is that the G414–A16 bond may be already broken in the transition state. In either case, the results suggest the "transesterification step" is actually more than one step, with the rate-limiting step involving a conformational change.

Analysis of the kinetics of linearization of C IVS at 42 and 50 °C is complicated by the presence of a second exponential component (see Figure 5). The origin of this second exponential has not been identified. One possible origin is linearization of C IVS by OH^- (Zaug et al., 1984, 1985). If this second reaction is not coupled to reverse cyclization by CU, then it should be possible to predict k_{obsd} for the faster exponential from the results presented above. The predicted values of k_{obsd} at 42 and 50 °C are 9 and 53 h^{-1}, respectively. The measured values from a double-exponential fit are 23 and 100 h^{-1}, respectively. If the curves at 42 and 50 °C are truncated at 120 and 20 min, respectively, and fit to a single exponential, the values derived for k_{obsd} are 13 and 60 h^{-1}, respectively. Considering the uncertainties, the results suggest the mechanism for reverse cyclization at 42 and 50 °C is the same as, or at least very similar to, the mechanism between 25 and 35 °C. The k_{obsd} measured at 42 °C can also be compared to the k_{obsd} of 26 h^{-1} measured by Sullivan and Cech (1985) for 1 mM UUU at 42 °C in 100 mM $(NH_4)_2SO_4$/10 mM Mg^{2+}, pH 7.5. This comparison suggests the different buffers do not have a large effect on the reaction rate.

Binding of the Mg^{2+} Is Unusually Weak. In mechanism 5, K_a represents the binding constant for a Mg^{2+} ion to the C IVS·CU complex. Thermodynamic parameters for this binding can be estimated from the temperature dependence of K_a. A van't Hoff plot of the data from Table I is shown in Figure 8. The $\Delta H°$ and $\Delta S°$ are −3 kcal/mol and 2 eu, respectively. Thus, the binding constant is only slightly dependent on temperature and is ~500 near 30 °C. These values can be compared with parameters for Mg^{2+} binding to other RNAs as listed in Table III. The small $\Delta H°$ and $\Delta S°$ are common for Mg^{2+} binding to RNA (Bujalowski et al., 1986; Romer & Hach, 1975; Rialdi et al., 1972; Krakauer, 1971). The small K_a, however, is less common. At 10 mM Na^+, it is more than 10 times smaller than the constant for binding of Mg^{2+} to weak binding sites on tRNA (Rialdi et al., 1972). It is within a factor of 10, however, of the constant for Mg^{2+} binding to $A(pA)_4$ in 1 mM Na^+ (Pörschke, 1979) and to the anticodon loop of tRNA in 100 mM Na^+ (Bujalowski et al., 1986). Both these complexes involve an inner-sphere coordination of Mg^{2+} that is very dependent on sequence. Interestingly, a recent three-dimensional model of this self-splicing RNA has several single stranded A_n sequences in the active site (Kim & Cech, 1987). The binding constant for such inner-sphere complexes will likely decrease with increasing ionic strength. According to eq 6, this would lead to a decrease in k_{obsd} as ionic strength is increased. A decrease in k_{obsd} is observed when the Na^+ concentration is increased from 10 to 100 mM (see Figure 1 and supplementary material). Thus, the kinetic results are consistent with a step that requires a Mg^{2+} ion to be specifically bound to one or more nucleotides in the C IVS·CU complex. Because of the specific binding, such a Mg^{2+} could function as a catalytic site.

The hypothesis that a weakly bound Mg^{2+} is required for catalysis also provides a potential rationale for some effects observed with recently constructed mutants of IVS. Burke et al. (1986) report that splicing activity is lost at or below 2 mM Mg^{2+} when certain mutations are made in elements R or S of the conserved P7 helix. Activity is restored, however, if the Mg^{2+} concentration is 10 mM or higher. It is likely that the equilibrium constant for inner-sphere complexation of a Mg^{2+} will be dependent on the three-dimensional structure of the RNA. For example, the Mg^{2+} bound to the anticodon loop of tRNA is coordinated to one oxygen of phosphate 37 and five water molecules. The five water molecules, however, hydrogen bond to atoms from five different nucleotides (Teeter et al., 1980). Thus, mutations that affect the structure of the RNA can change the binding constant for the Mg^{2+} ion. In this model, higher Mg^{2+} concentrations would simply compensate for this lower binding constant by ensuring the catalytic site contained an inner-sphere-complexed Mg^{2+}.

When the kinetic point at 0.5 mM Mg^{2+} in Figure 3 is included in the fit to eq 6, a negative value is obtained for k_{-b}. This suggests that, below 1 mM, Mg^{2+} has additional effects on the circle-opening reaction. This is not surprising since presumably there will be many binding sites for Mg^{2+} on C IVS, and the occupancy of these sites will affect structure and possibly the active site. Thus, as the Mg^{2+} concentration is lowered, additional effects will be observed.

The dependence of the observed rate on the concentrations of CU and Mg^{2+} is consistent with either of the following mechanisms:

$$\text{C IVS} + \text{CU} \rightleftharpoons \text{C IVS·CU} \xrightleftharpoons{+Mg^{2+}} Mg^{2+}\text{·C IVS·CU} \rightleftharpoons \text{CU-L'IVS·}Mg^{2+} \quad (8)$$

$$\text{C IVS} + Mg^{2+} \rightleftharpoons Mg^{2+}\text{·C IVS} \xrightleftharpoons{+CU} Mg^{2+}\text{·C IVS·CU} \rightleftharpoons \text{CU-L'IVS·}Mg^{2+} \quad (9)$$

Some advantages can be envisioned for adding an inner-sphere-bound Mg^{2+} ion after the CU substrate. If substrate binding is required to make the Mg^{2+} site available, then

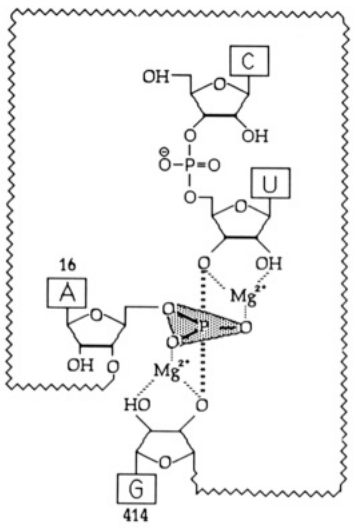

FIGURE 9: Schematic of potential transition state for reverse cyclization. The proposed transition state involves coordination between Mg^{2+} ions and ribose hydroxyl groups from G414 and U of CU. The model represents one possibility for a Mg^{2+} binding site that would be stronger in the presence of CU.

competing reactions, such as hydrolysis by OH^- (Zaug et al., 1984), will be suppressed. This would enhance the specificity of the reaction. This is an important consideration for the intervening sequence since in exon splicing it must choose 2 out of 6000 bonds for cleavage.

One intriguing possibility is that the weakly bound Mg^{2+} is coordinated to the uridine residue of the CU substrate. A drawing of one such transition state is shown in Figure 9. The coordination of the Mg^{2+} bound to the uridine is similar to that recently proposed from theoretical considerations by Toh et al. (1987). There are several attractive features for this working hypothesis. Coordination of Mg^{2+} to CU could provide additional binding energy that would partially account for the anomalously large binding constant observed for CU. A similar Mg^{2+} could provide extra binding energy for the G cofactor. The transition state is accessible from both C IVS CU and CU-L′IVS, so the reaction is reversible as observed (Sullivan & Cech, 1985). With L′IVS made from linearization by OH^-, however, the Mg^{2+} binding site created by coordination to the 2′- and 3′-hydroxyls of U would be absent. Thus, binding of Mg^{2+} at this catalytic site would be much weaker. This could be one factor accounting for the fact that the reverse of circle opening by OH^- has not been observed at 10 mM Mg^{2+} (Zaug et al., 1985).

The results reported here indicate the mechanism for reverse cyclization involves separate steps for binding CU and Mg^{2+}. The sequence for those binding steps can be determined from transient kinetic studies. Such studies may also reveal additional steps in the mechanism. Reaction from the intermediate with CU and Mg^{2+} bound is associated with a large activation energy. Thus, this intermediate can be easily trapped and studied. For example, the results predict the half-life for reaction from this intermediate is about 3 days at 4 °C. Thus, it should be possible to test many aspects of the mechanistic hypotheses suggested by these initial experiments.

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SUPPLEMENTARY MATERIAL AVAILABLE

Seven figures showing time courses of reaction of oligomers with C IVS and one figure showing k_{obsd} vs [UCU] at 30 °C (8 pages). Ordering information is given on any current masthead page.

APPENDIX

One simple derivation of the kinetic equations is presented. The mechanism is

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} D \qquad (A1)$$

Let C_A, C_B, C_C, and C_D be the concentrations of A, B, C, and D, respectively, at any time, t; $\bar{C}_A$, $\bar{C}_B$, $\bar{C}_C$, and $\bar{C}_D$ be the equilibrium concentrations; ΔC_A, ΔC_B, ΔC_C, and ΔC_D be the displacements from equilibrium at any time. Thus, $\Delta C_A = C_A - \bar{C}_A$, $\Delta C_D = C_D - \bar{C}_D$, etc.

It will be useful to have a relationship between ΔC_A and ΔC_D. If the first step in the mechanism is fast enough that A, B, and C can be considered always in equilibrium, then

$$K_1 = \frac{k_1}{k_{-1}} = \frac{\bar{C}_C + \Delta C_C}{(\bar{C}_A + \Delta C_A)(\bar{C}_B + \Delta C_B)} \qquad (A2)$$

$$K_1(\bar{C}_A\bar{C}_B + \bar{C}_A\Delta C_B + \bar{C}_B\Delta C_A + \Delta C_A\Delta C_B) = \bar{C}_C + \Delta C_C \qquad (A3)$$

By definition, $K_1 = \bar{C}_C/\bar{C}_A\bar{C}_B$. If $C_B \gg C_A$, then $\Delta C_A\Delta C_B \ll \bar{C}_B\Delta C_A$, so terms in $\Delta C_A\Delta C_B$ can be neglected. From stoichiometry, $\Delta C_A = \Delta C_B$. This leads to

$$\Delta C_C = K_1(\bar{C}_A + \bar{C}_B)\Delta C_A \qquad (A4)$$

Again from stoichiometry, $\Delta C_A = -\Delta C_C - \Delta C_D$, which leads to the relationship between ΔC_A and ΔC_D

$$\Delta C_A = -\Delta C_D/[K_1(\bar{C}_A + \bar{C}_B) + 1] \qquad (A5)$$

For mechanism A1, the rate of product formation is

$$dC_D/dt = d\Delta C_D/dt = k_2C_C - k_{-2}C_D = k_2(\bar{C}_C + \Delta C_C) - k_{-2}(\bar{C}_D + \Delta C_D) \qquad (A6)$$

At equilibrium, $d\Delta C_D/dt = 0 = k_2\bar{C}_C - k_{-2}\bar{C}_D$. From stoichiometry, $\Delta C_C = -\Delta C_A - \Delta C_D$, leading to

$$d\Delta C_D/dt = k_2\Delta C_C - k_{-2}\Delta C_D = -k_2\Delta C_A - k_2\Delta C_D - k_{-2}\Delta C_D \qquad (A7)$$

Expressing ΔC_A in terms of ΔC_D gives

$$\frac{d\Delta C_D}{dt} = \left[\frac{k_2}{K_1(\bar{C}_A + \bar{C}_B) + 1} - k_2 - k_{-2}\right]\Delta C_D = -\left[\frac{k_2K_1(\bar{C}_A + \bar{C}_B)}{K_1(\bar{C}_A + \bar{C}_B) + 1} + k_{-2}\right]\Delta C_D \qquad (A8)$$

When $C_B \gg C_A$, then $(\bar{C}_A + \bar{C}_B) \approx C_B^0$, the initial concentration of C_B. Then

$$\frac{d\Delta C_D}{dt} = -[\{k_2C_B^0/(C_B^0 + 1/K_1)\} + k_{-2}]\Delta C_D \qquad (A9)$$

Letting the expression in brackets be k_{obsd}, rearranging, and integrating in the standard way for first-order reactions gives

$$\int_{\Delta C_D^0}^{\Delta C_D^t} \frac{d\Delta C_D}{\Delta C_D} = \ln \Delta C_D^t - \ln \Delta C_D^0 = -k_{obsd}\int_0^t dt = -k_{obsd}t \qquad (A10)$$

$$\Delta C_D^t = \Delta C_D^0 e^{-k_{obsd}t} \qquad (A11)$$

Here ΔC_D^t is the displacement of D from equilibrium at time t. It is related to the fraction of D present at any time, f_t, by being proportional to $f_\infty - f_t$. For experiments in which $C_D = 0$ at $t = 0$, $\Delta C_D^0 \propto f_\infty$. Thus

$$f_\infty - f_t = f_\infty e^{-k_{obsd}t} \quad \text{(A12)}$$

$$f_t = f_\infty(1 - e^{-k_{obsd}t}) \quad \text{(A13)}$$

Registry No. CU, 2382-64-1; UCU, 6802-32-0; CUCU, 74145-00-9; CUCUCU, 115338-97-1; Mg, 7439-95-4.

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