Sarma, R. H., Eds.) p 227, Adenine Press, New York.
Mirau, P. A., & Kearns, D. R. (1984a) *J. Mol. Biol. 177*, 207–227.
Mirau, P. A., & Kearns, D. R. (1984b) *Biochemistry 23*, 5439–5446.
Mirau, P. A., & Kearns, D. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1594–1598.
Nordheim, A., Lafer, E. M., Peck, L. J., Wang, J. C., Stollar, D., & Rich, A. (1982) *Cell (Cambridge, Mass.) 31*, 309–318.
Palecek, E. (1969) *Prog. Nucleic Acid Res. Mol. Biol. 9*, 31–74.
Patel, D. J. (1978) *J. Polym. Sci. 62*, 117–141.
Patel, D. J., Canuel, L., & Pohl, F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2508–2511.
Patel, D. J., Kozlowski, S. A., Nordheim, A., & Rich, A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1413–1417.
Pilet, J., & Leng, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 26–30.
Pohl, F. M., & Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375–396.
Rich, A., Nordheim, A., & Wang, H.-J. (1984) *Annu. Rev. Biochem. 53*, 791–846.
Robinson, B. H., Lerman, L. S., Beth, A. H., Frisch, H. L., Dalton, L. R., & Auer, C. (1980) *J. Mol. Biol. 139*, 19–44.
Sarocchi, M. Th., & Guschlbauer, W. (1973) *Eur. J. Biochem. 34*, 232.
Sobell, H. M., Tsai, C.-C., Gilbert, S. G., Jain, S. C., & Sakore, T. D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3068–3072.
Taylor, R., & Kennard, O. (1982) *J. Am. Chem. Soc. 104*, 3209–3212.
Teitelbaum, H., & Englander, S. W. (1975a) *J. Mol. Biol. 92*, 55–78.
Teitelbaum, H., & Englander, S. W. (1975b) *J. Mol. Biol. 92*, 79–92.
Thomas, J. C., & Schurr, J. M. (1983) *Biochemistry 22*, 6194–6198.
Thomas, T. J., & Bloomfield, V. A. (1983) *Nucleic Acids Res. 11*, 1919–1930.
Tirado, M. M., & Garcia de la Torre, J. (1980) *J. Chem. Phys. 73*, 1986–1993.
Tjerneld, F. (1982) Ph.D. Thesis, Chalmers University of Technology, Goteborg, Sweden.
Wang, A. H. J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., Van Boom, J. H., Van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.
Wang, A. H. J., Quigley, G., Kolpak, F., Van der Marel, G., Van Boom, J., & Rich, A. (1981) *Science (Washington, D.C.) 211*, 171–176.
Williams, D. R. (1972) *Chem. Rev. 72*, 203–213.
Wing, R., Drew, H., Takano, T., Broka, C., Tanaka, S., Itakura, K., & Dickerson, R. E. (1980) *Nature (London) 287*, 755–758.
Wittebort, R. J., & Szabo, A. (1978) *J. Chem. Phys. 69*, 1722–1736.
Woessner, D. E. (1962) *J. Chem. Phys. 37*, 647–654.
Woessner, D. E., Snowden, B. S., & Meyer, G. H. (1969) *J. Chem. Phys. 50*, 719–721.
Wright, J. M., Feigon, J., Denny, W. A., Leupin, W., & Kearns, D. R. (1981) *J. Magn. Reson. 45*, 514–519.
Wu, H. M., Dattagupta, N., & Crothers, D. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6808–6811.

Reactions of the Intervening Sequence of the *Tetrahymena* Ribosomal Ribonucleic Acid Precursor: pH Dependence of Cyclization and Site-Specific Hydrolysis†

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ABSTRACT: During self-splicing of the *Tetrahymena* rRNA precursor, the intervening sequence (IVS) is excised as a unique linear molecule and subsequently cyclized. Cyclization involves formation of a phosphodiester bond between the 3′ end and nucleotide 16 of the linear RNA, with release of an oligonucleotide containing the first 15 nucleotides. We find that the rate of cyclization is independent of pH in the range 4.7–9.0. A minor site of cyclization at nucleotide 20 is characterized. Cyclization to this site becomes more prominent at higher pHs, although under all conditions examined it is minor compared to cyclization at nucleotide 16. The circular IVS RNAs are unstable, undergoing hydrolysis at the phosphodiester bond that was formed during cyclization. We find that the rate of site-specific hydrolysis is first order with respect to hydroxide ion concentration, with a rate constant 10^3–10^4-fold greater than that of hydrolysis of strained cyclic phosphate esters. On the basis of these results, we propose that circular IVS RNA hydrolysis involves direct attack of OH^- on the phosphate at the ligation junction, that particular phosphate being made particularly reactive by the folding of the RNA molecule. Cyclization, on the other hand, appears to occur by direct attack of the 3′-terminal hydroxyl group of the linear IVS RNA without prior deprotonation.

Splicing of the rRNA precursor of *Tetrahymena* occurs by a series of transesterification (phosphoester transfer) reactions (Cech et al., 1981; Cech, 1983). The phosphodiester bond energy is conserved in such reactions, which explains the lack of a requirement for ATP or GTP hydrolysis. Splicing is mediated by the structure of the RNA and requires no enzyme or other protein, at least in vitro (Kruger et al., 1982). It now appears that mitochondrial rRNA splicing in *Neurospora* and yeast follows the transesterification mechanism (Garriga &

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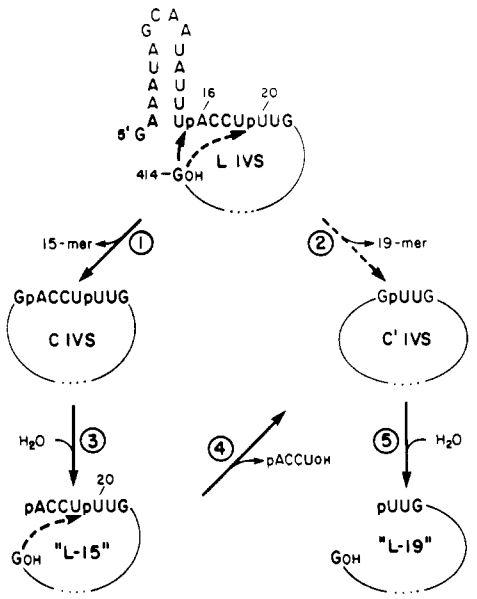


FIGURE 1: Model for the IVS RNA cyclization and hydrolysis reactions (Zaug et al., 1984). (1) Predominant cyclization reaction. (2) Minor cyclization reaction. (3) Reopening of the C IVS by site-specific hydrolysis, which reestablishes the 3′-terminal nucleophile (G-OH). (4) Recyclization of the L − 15 RNA to the secondary cyclization site. (5) Site-specific hydrolysis of C′ IVS, the smaller circle. Evidence for reaction 1 is presented in Zaug et al. (1983), for reaction 2 in this work, and for reactions 3–5 in Zaug et al. (1984).

Lambowitz, 1983; Tabak et al., 1984), and there is reason to believe that much of fungal mitochondrial mRNA splicing may be mechanistically similar [reviewed by Cech (1983)]. In addition, a cytochrome *b* mRNA intron from *Neurospora* mitochondria (Garriga & Lambowitz, 1984) and the rRNA intron from yeast mitochondria (Van der Horst & Tabak, 1985) have recently been found to undergo self-splicing in vitro.

While the transesterification mechanism is not restricted to *Tetrahymena* rRNA splicing, it is certainly not general for all RNA splicing. Transfer RNA splicing in yeast and wheat occurs by a quite different mechanism involving ATP hydrolysis and is catalyzed by two enzymes, an endonuclease and a ligase [e.g., see Greer et al. (1983) and Konarska et al. (1982)]. Splicing of nuclear pre-mRNA, on the other hand, occurs by formation of a branched RNA intermediate (Wallace & Edmonds, 1983; Padgett et al., 1984; Ruskin et al., 1984). As noted by Padgett et al. (1984), the mechanism could involve two transesterification reactions or could involve separate cleavage and ligation reactions catalyzed by enzymes similar to those found in bacteria.

In *Tetrahymena*, the excised intervening sequence (IVS)[1] RNA undergoes autocyclization both in vitro (Grabowski et al., 1981) and in vivo (Brehm & Cech, 1983). Cyclization is not a simple end-to-end ligation of the linear IVS RNA molecule. Instead, it involves joining of the 3′-terminal guanosine of the linear IVS to the nucleotide at position 16, with the concomitant release of an oligonucleotide containing the first 15 nucleotides of the linear molecule (Figure 1; Zaug et al., 1983). Thus, IVS cyclization, like RNA splicing, occurs by transesterification. Cyclization is a good model system for studying the properties of such reactions, because the linear IVS RNA is a homogeneous molecular species that can be obtained in large amounts. RNA cyclization has proven useful in the study of a variety of other RNA ligation activities (Silber et al., 1972; Bruce & Uhlenbeck, 1978; Konarska et al., 1981).

We recently found that the circular IVS RNA contains a single labile phosphodiester bond, the one that was formed during cyclization (Zaug et al., 1984). Hydrolysis at this site produces the "L − 15" RNA (the linear IVS minus its first 15 nucleotides), which has 5′-phosphate and 3′-hydroxyl termini; such termini are unusual products for chemical hydrolysis of RNA but typical of the other reactions mediated by this molecule. The recreated 3′-terminal guanosine undergoes another round of cyclization (to the nucleotide at position 20) and reopening, resulting in an "L − 19" IVS RNA (Figure 1; Zaug et al., 1984). Circle hydrolysis is a slow reaction in vitro, probably too slow to contribute to RNA metabolism in vivo. Nevertheless, the reaction provides important insight as to how an RNA molecule can be folded to strain or activate a specific phosphate at the site of an RNA processing reaction.

In our initial study (Zaug et al., 1984), we found that circle reopening occurred much faster at pH 9.0 than at pH 7.5. We now present a more detailed study of the pH dependence of the hydrolysis reaction and compare it to the pH dependence of the IVS cyclization reaction. On the basis of the different pH dependence of these two reactions, we propose a specific mechanism for circle reopening. In the course of these studies, we found that the initial cyclization reaction is not absolutely precise. A small proportion of the L IVS undergoes cyclization at nucleotide 20, the same site that is involved in cyclization of the L − 15 RNA.

Experimental Procedures

Synthesis and Purification of IVS RNA. RNA was synthesized in vitro by transcription of an *Escherichia coli* plasmid with purified *E. coli* RNA polymerase. The plasmid, pIVS11, has an IVS-containing restriction fragment of the *Tetrahymena thermophila* rDNA inserted downstream from the *lac* UV5 promoter (Kruger et al., 1982). To obtain L IVS RNA, pre-rRNA was synthesized with the *Tetrahymena* nuclear transcription buffer described by Kruger et al. (1982). After a 30-min transcription at 30 °C, the $(NH_4)_2SO_4$ concentration was increased to 120 mM, and incubation was continued for an additional 30 min at 30 °C to promote self-splicing. A revised protocol was used to obtain C IVS RNA. Plasmid DNA (20 μg) was incubated for 1 h at 37 °C in 500 μL of a solution that consisted of 250 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM 2-mercaptoethanol, 1 mM spermidine, 1 mM putrescine, 0.1 mM spermine, 50 mM Tris-HCl, pH 8.0, 0.5 mM each unlabeled nucleoside triphosphate, 0.1 mM each nucleoside [α-^{32}P]triphosphate (80 μCi/500 μL, 760 Ci/mmol), and 0.2 μM *E. coli* RNA polymerase. ATP, the initiating nucleotide, was always used at the 0.5 mM concentration. (At this elevated NaCl concentration, efficient excision of the IVS occurs during the transcription reaction. At the temperature of 37 °C, a mixture of L and C IVS RNAs is obtained.) The nucleic acids were purified by extraction with phenol and chloroform followed by ethanol precipitation and were then subjected to electrophoresis in a 4% polyacrylamide–8 M urea gel. The gel was stained with ethidium bromide, and the bands corresponding to the L and C IVS RNAs were cut out. The RNA was eluted from the gel slices by crushing and soaking overnight according to the procedure

[1] Abbreviations: IVS, intervening sequence; L IVS RNA, the linear intervening sequence (414 nucleotides) that is the direct product of pre-rRNA splicing; C IVS RNA, the major circular form (399 nucleotides); L − 15 RNA (read "L minus 15"), a 399-nucleotide linear form missing the first 15 nucleotides of the L IVS; L − 19 RNA, a 395-nucleotide linear form missing the first 19 nucleotides of the L IVS; Tris, tris(hydroxylmethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; HEPPS, *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid.

of Peattie (1979). The absence of Mg^{2+} from the crush-and-soak solution was necessary to prevent cyclization of L IVS RNA and reopening of C IVS RNA during their isolation.

5′-End-Labeled L IVS RNA. pIVS11 was transcribed as described above, except that the NaCl concentration was limited to 25 mM, the time to 30 min, and the temperature to 30 °C. Under these conditions the excision of IVS RNA during transcription was minimized. [^{3}H]UTP (250 μCi/mL) was substituted for the ^{32}P-labeled nucleotide. After purification, the nucleic acids were subjected to chromatography on Sephadex G-50 (in 0.25 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl, pH 7.5 and 0.1% sodium dodecyl sulfate) to remove unincorporated nucleotides. The [^{3}H]RNA was precipitated with 3 volumes of ethanol. Splicing and concomitant labeling of the 5′ end of the excised IVS were accomplished by incubation of the RNA for 30 min at 30 °C in 10 μM [α-^{32}P]GTP (760 Ci/mmol), 5 mM $MgCl_2$, 200 mM NaCl, and 10 mM Tris-HCl, pH 8.0. The ^{32}P-labeled L IVS RNA was purified by gel electrophoresis as described above.

Cyclization Reactions. Standard reaction solutions (15 μL) were 2.5–2.8 nM L IVS, 10 mM $MgCl_2$, and 50 mM buffer. Solutions containing RNA and buffer were equilibrated at 42 °C for 2 min. Reactions were initiated by the addition of $MgCl_2$ (which was determined not to affect the pH). Reactions were stopped on ice by the addition of EDTA and Tris-HCl, pH 7.5, to give final concentrations of 30 and 100 mM, respectively. The extent of cyclization was assayed by electrophoresis in a 4% polyacrylamide–8 M urea gel, drying of the gel, and autoradiography, as described by Cech et al. (1981). Quantitation of each dried gel was accomplished by cutting out regions that corresponded to the linear (L IVS and L – 15 IVS) and circular IVS RNAs and blank regions of equal area (for background determination) by using the autoradiogram as a template. ^{32}P radioactivity was determined by liquid scintillation counting in a toluene-based fluor. In those lanes that contained both L IVS and L – 15 IVS RNAs, the percentage of radioactivity in each species was determined from the peak heights of a spectrophotometric scan of the autoradiogram. Having proven that the L – 15 IVS RNA was derived from reopening of C IVS RNA, we added the percentages of L – 15 and C IVS to obtain the total percent cyclization.

Circle Reopening (Site-Specific Hydrolysis) Reactions. C IVS RNA was incubated at 42 °C in 10 mM $MgCl_2$, 200 mM NaCl, and 50 mM buffer. Reactions were stopped by the addition of EDTA to a final concentration of 20 mM. Products were separated by gel electrophoresis, and the extent of circle reopening was determined by liquid scintillation counting as for the cyclization reactions.

RNA Sequence Analysis. End-labeled L IVS RNA and oligonucleotides released during cylization (the 15-mer and 19-mer) were purified by gel electrophoresis. They were sequenced according to the procedures of Donis-Keller et al. (1977) and Donis-Keller (1980), with slight modifications. L IVS RNA in a solution containing 0.25 μg/μL tRNA was incubated for 30 min, 50 °C, at pH 3.5 with 1×10^{-4} unit/μL RNase T_1 or RNase U_2 or at pH 5.0 with 0.4 unit/μL RNase Phy M. Partial alkaline hydrolysis was for 6 min at 95 °C. The 15-mer and 19-mer were similarly incubated with 1×10^{-3} unit/μL RNase T_1 or RNase U_2 or with 0.8 unit/μL RNase Phy M. Partial alkaline hydrolysis was for 15 min at 95 °C. RNases T_1 and U_2 were purchased from Calbiochem, and RNase Phy M was from Bethesda Research Laboratories.

Results

pH Dependence of IVS RNA Cyclization. Purified L IVS was incubated at 42 °C in buffered solutions containing 10 mM $MgCl_2$. Cyclization occurred with $t_{1/2} \simeq 1$ min at pH 7.5. A 2-min reaction time was therefore chosen so that the extent of reaction would be sensitive to changes in cyclization rate at other pHs. Cyclization was monitored by electrophoresis of the RNA in a denaturing polyacrylamide gel and autoradiography, as shown in Figure 2A,B. There was no detectable cyclization at pH <4.0, at which pHs the RNA is probably denatured. The extent of cyclization at 2 min was constant between pH 4.7 and pH 8.5. Between pH 8.5 and 10.0, there was a much larger extent of reaction of the L IVS RNA at 2 min. The reaction products included C IVS RNA and L – 19 RNA (linear molecules migrating slightly faster than the original L IVS RNA). In a separate study, we have shown that the L – 19 RNA is derived from C IVS RNA by the reaction scheme shown in Figure 1. Incubation of purified C IVS RNA at pH 9.4 showed that the rate of circle reopening under these conditions was high enough to account for all of the L – 19 RNA being derived from C IVS RNA (data not shown). Thus, in Figure 2B the amount of cyclization is taken as the sum of the C IVS and L – 19 IVS RNA species. The amount of cyclization decreased again at pH >10.

To investigate whether the increased extent of reaction at 2 min at pH 9 was due to a faster cyclization rate, kinetic studies were performed (Figure 2C). Surprisingly, cyclization was found to be better at pH 9 not because of a faster initial rate but because there was a larger fraction of the molecules in the fast kinetic component. When the data were normalized to the amount of reaction at 10 min and plotted as log fraction L IVS RNA vs. time, the pH 7.5 and pH 9.0 graphs were indistinguishable (not shown). In each case the fast component had a $t_{1/2} = 0.9 \pm 0.3$ min (mean ± range). From other studies, we know that the extent of cyclization at pH 7.5 could be increased by addition of monovalent cations (unpublished results). We therefore compared the rates of cyclization at pH 7.5 and pH 9.0 in the presence of 200 mM NaCl. As shown in Figure 2D, the magnitude of the pH effect was diminished somewhat, but the effect remained one of extent of reaction rather than rate. At both pHs the fast kinetic component had a $t_{1/2} = 1.0 \pm 0.3$ min.

Two Sites for the Cyclization Reaction. One possible explanation for the increased extent of cyclization at high pH was that the L IVS that did not cyclize at pH 7.5 was able to cyclize via a different reaction at pH 9. We therefore examined the site of IVS cyclization as a function of pH. 5′-End-labeled L IVS RNA was incubated under various conditions, and the size of the 5′-terminal oligonucleotide released during cyclization was determined by sequencing gel electrophoresis (Figure 3). On the basis of the amount of L IVS remaining at 30 min, it was again apparent that the extent of cyclization increased with pH and, around neutral pH, with the addition of 200 mM NaCl. Under all conditions examined, the major labeled oligonucleotide was the 15-mer characterized previously (Zaug et al., 1983). In addition, a 19-nucleotide fragment was produced to some extent in all reactions; this 19-mer is described below. A 26-nucleotide fragment was observed in those reactions that contained Tris buffer. It increased in amount between 10 and 50 mM Tris. The production of this fragment was variable among preparations of IVS RNA. Much less 26-mer was produced in other RNA preparations than in that shown in Figure 3. The possible origin of the 26-mer will be considered under Discussion.

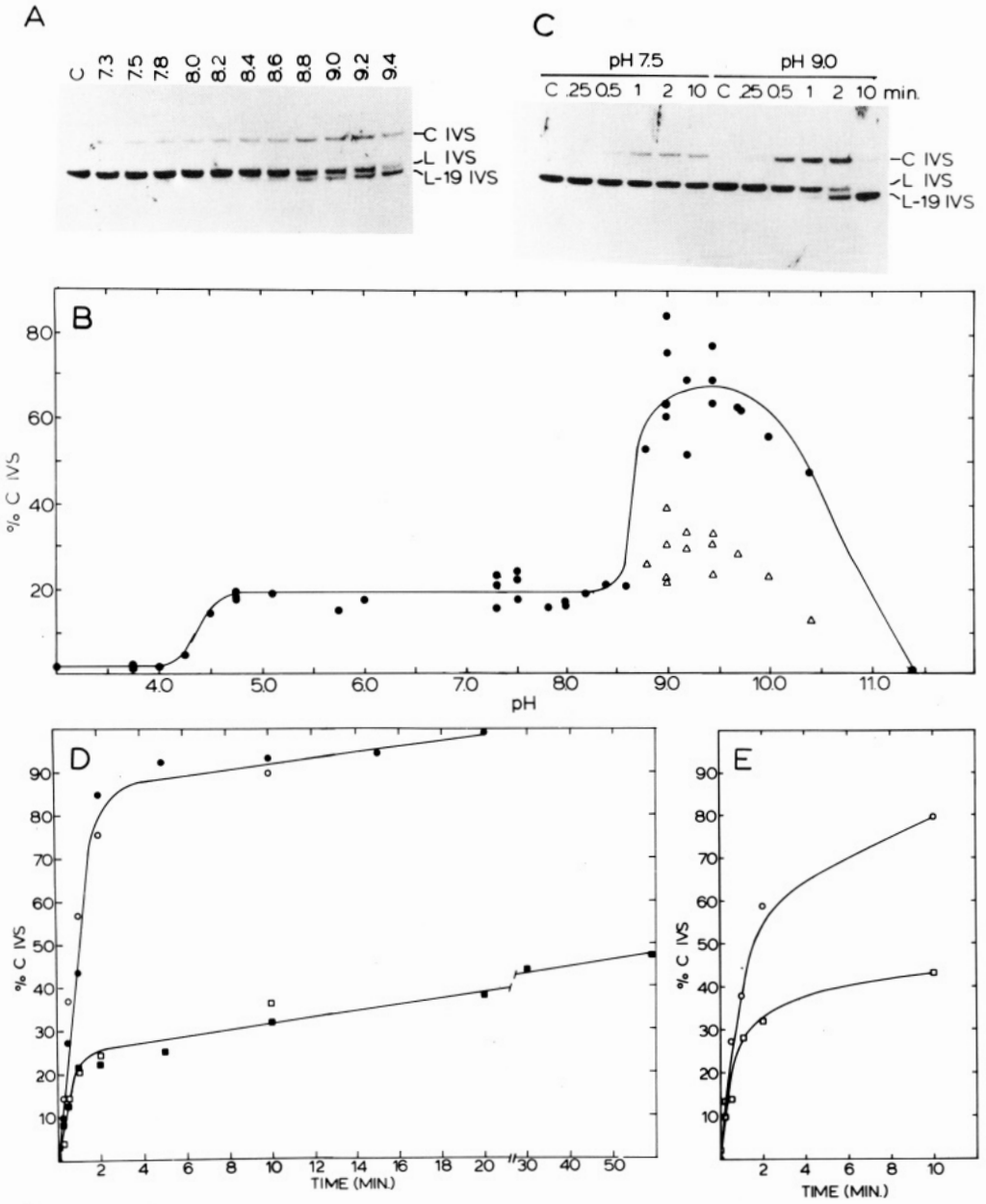


FIGURE 2: Effect of pH on L IVS RNA cyclization. (A) Extent of cyclization after 2 min under standard cyclization conditions as assayed by 4% polyacrylamide–8 M urea gel electrophoresis. A sample of the data in the pH range 7.3–9.4 is shown. (Lane C) Incubation on ice; pH 8.4 buffer. (B) Quantitation of several gels showing the effect of pH on extent of cyclization after a 2-min reaction. Buffers used were formate (pK_a = 3.75) at pH 3.0–4.75, acetate (pK_a = 4.75) at pH 4.75–5.75, MES (pK_a = 6.15) at pH 5.10–7.30, Tris (pK_a = 8.30) at pH 7.30–9.40, and CAPS (pK_a = 10.40) at pH 9.40–11.40. For Tris buffer, the pH was adjusted for the reaction temperature at 42 °C. (●) IVS RNA that had undergone cyclization, taken as 100(C IVS + L – 19 IVS)/(L IVS + C IVS + L – 19 IVS). (△) Percent C IVS RNA only; reopened circle was present at these pHs. (C) Kinetics of cyclization in 10 mM $MgCl_2$–50 mM Tris-HCl at pH 7.5 and at pH 9.0. (Lanes C) Incubation on ice. (D) Quantitation of data including that shown in (C). (○) pH 9.0. (□) pH 7.5. The shaded data points represent a repeated set of experiments. (E) Kinetics of cyclization in the presence of 200 mM NaCl. (○) pH 9.0. (□) pH 7.5.

Sequence analysis of the 19-mer confirmed that it contains the first 19 nucleotides of the L IVS RNA (Figure 4). Thus, it is the product of splicing at the normal site followed by a cleavage reaction (presumably accompanying cyclization) at the U_{19}–U_{20} bond, as opposed to splicing at a minor site 4 nucleotides upstream from the normal 5′-splice site followed by cyclization at the normal U_{15}–A_{16} bond. The proportion of reaction at U_{20} depended on the cyclization conditions in vitro. For example, in 10 mM $MgCl_2$–50 mM Tris-HCl the proportion of 19-mer increased with pH in the presence or absence of 200 mM NaCl (Figure 3A). In other experiments the radioactivity in the various species was quantitated by using liquid scintillation counting. With reactions carried out at 42 °C in 10 mM $MgCl_2$, the proportion of 19-mer [calculated as 19-mer/(15-mer + 19-mer)] was 0.04 at pH 7.5 and 0.20 at pH 9.0.

pH Dependence of Circular IVS Hydrolysis. Purified C IVS RNA was incubated for various amounts of time at 42 °C. Hydrolysis of the circle was assayed by its conversion to the linear form, as determined by polyacrylamide gel electrophoresis and autoradiography (Figure 5A). At a given pH, hydrolysis of the C IVS RNA followed pseudo-first-order kinetics (Figure 5B). The pseudo-first-order rate constant k_{obsd} increased linearly with pH up to pH 9.5 (Figure 5C). The reaction kinetics can be described by

$$-d[\text{C IVS}]/dt = k[OH^-][\text{C IVS}]$$

where $k = k_{obsd}/[OH^-] = 1700 \pm 230$ min^{-1} M^{-1} at 42 °C

(mean ± SD of the values of k for pH 7.5–9.0).[2] The calculation of the second-order rate constant is based on activities.

The rate of circle reopening was lower at pH 10.0 than at pH 9.5. This may reflect a loss of reactivity due to denaturation of the RNA, since both uridine and guanosine become deprotonated in this pH range. Alkaline hydrolysis resulted in substantial degradation of the RNA at pH ≥10.5, making it difficult to collect reliable data.

Discussion

Mechanism of IVS RNA Cyclization. The rate of the cyclization reaction is independent of pH in the range 4.7–9.0. The lack of a pH dependence is most easily interpreted as an indication that the nonionized 3′-hydroxyl group of G_{414} is the reactive species. The proton would then be transferred to solvent, to the 2′-OH of G_{414}, or to some other base that does not undergo ionization in the pH range that was examined. Possible candidates for such a base would be the N-3 of a cytidine (pK_a = 4.5), the N-1 of an adenosine (pK_a = 3.7), or the N-7 of a guanosine [pK_a = 2.4; pK_a values for nucleoside 5′-phosphates at 25 °C from Ts'o (1974)]. An alternative explanation is that the anionic form of G_{414} is the reactive species but that the pH dependence of deprotonation of the 3′-hydroxyl is compensated by the pH dependence of protonation of, for example, the N-3 of cytidine. This seems unattractive because the ribose sugar is such a weak acid ($pK_a \simeq 12.4$) and the nucleic acid bases are such weak bases. Thus, the concentration of the hypothetical reactive species would be exceedingly low.

It is more difficult to reconcile the data with the mechanism proposed in a theoretical paper by Haydock & Allen (1985). They propose that an OH^- ligand of a Mg^{2+} coordination complex acts as a specific base catalyst, serving to deprotonate the 3′-OH of G_{414}. The acid dissociation of Mg^{2+} ($Mg^{2+} + H_2O \rightleftarrows MgOH^+ + H^+$) has a pK = 11.44 ± 0.1 (Baes & Mesmer, 1976). One would therefore expect the occupancy of the Mg^{2+} coordination sphere by OH^- to increase linearly with [OH^-] throughout the pH range investigated in our study, with a concomitant increase in the rate of cyclization. This is contrary to the observed lack of pH dependence.

Although the rate of the reaction does not depend on pH, the fraction of the RNA that cyclizes in the rapid phase of the reaction does increase sharply at pH >8.8. At pH >8.8, more than 90% of the L IVS RNA cyclizes in a reaction that can be characterized with a single first-order rate constant, k = 0.8 min^{-1}. At pH <8.6, only 30% of the L IVS cyclizes at that rate. At neutral pH, the fraction of RNA that cyclizes in the rapid phase can be increased by addition of monovalent cations or formamide (50% formamide at 30 °C; A. J. Zaug, unpublished data). We propose that at low salt and neutral pH, more than half of the IVS RNA molecules are trapped in an unreactive conformation; for these molecules, undergoing a structural transition to attain the reactive conformation is the rate-limiting step in cyclization. Equilibration between the reactive and unreactive conformers is rapid under conditions of pH 9 or high salt or in the presence of the denaturant, formamide. The effect of elevated pH is unknown but may involve a titratable group on the RNA. Kao & Crothers

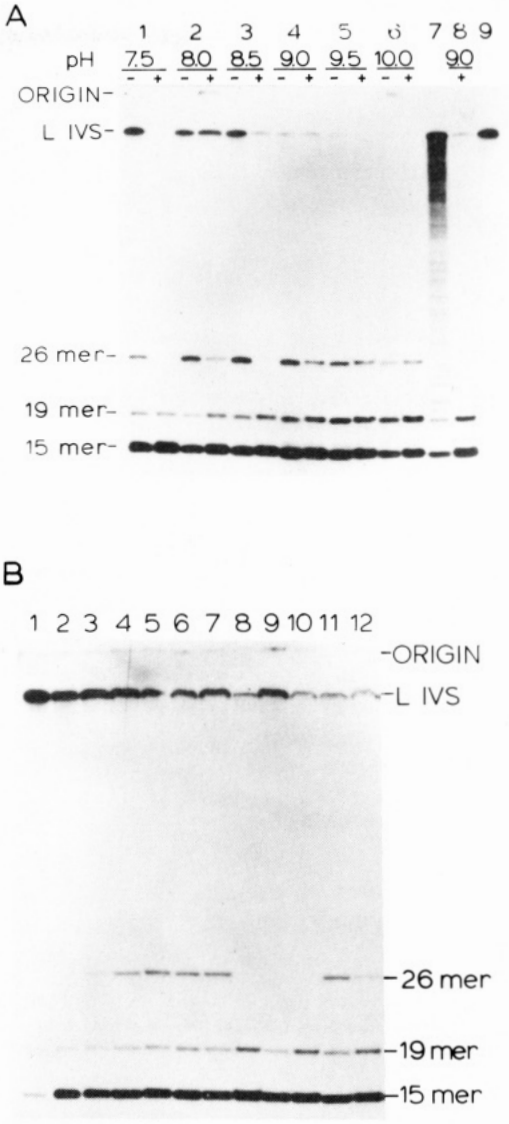


FIGURE 3: Oligonucleotides released from the 5′ end of the L IVS RNA during cyclization under various conditions. Reaction products were separated by electrophoresis in 20% polyacrylamide–7 M urea gels. (A) L IVS RNA, 5′ end labeled with [^{32}P]GTP, was incubated at 42 °C for 10 min at the indicated pH in (lanes 1–6) 10 mM $MgCl_2$–50 mM Tris-HCl or (lane 8) 10 mM $MgCl_2$–50 mM CHES. (+ lanes) An additional 200 mM NaCl was present in the reaction. (Lane 7) Limited alkaline hydrolysis of the L IVS RNA, serving as a marker for the electrophoretic mobilities of a complete series of oligonucleotides. (Lane 9) No incubation; the 15-mer in this sample is due to a small amount of previous cyclization in the preparation of the L IVS RNA. (B) Portions of the same preparation of L IVS RNA were (lane 1) not incubated or incubated at 42 °C for 10 min under the following conditions: (lane 2) 25 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, and 10 mM Tris-HCl, pH 7.5, as in Zaug et al. (1983); (lanes 3–7) 25 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, and 50 mM Tris-HCl at (lane 3) pH 7.5, (lane 4) pH 8.5, (lane 5) pH 9.0, (lane 6) pH 9.5, and (lane 7) pH 10.0; (lane 8) 10 mM $MgCl_2$–50 mM CHES, pH 9.0; (lane 9) same as lane 8 with the addition of 50 mM $(NH_4)_2SO_4$; (lane 10) same as lane 8 with the addition of 200 mM NaCl; (lane 11) 10 mM $MgCl_2$–50 mM Tris-HCl, pH 9.0; (lane 12) same as lane 11 with the addition of 200 mM NaCl.

[2] There is a small systematic error in the analysis of the rate of C IVS hydrolysis. As shown in Figure 1, the product of circle reopening, the L – 15 IVS RNA, forms another circle (C′ IVS) which reopens to form another linear molecule (L – 19 IVS). The C and C′ species differ by 4 nucleotides and are not resolved by gel electrophoresis. The same situation holds for the L – 15 and L – 19 species. Thus, the graphs in Figure 5 give (C + C′)/[C + C′ + (L – 15) + (L – 19)], whereas the extent of reaction would be properly evaluated as the fraction of C IVS RNA remaining, C/[C + C′ + (L – 15) + (L – 19)]. The fraction of unreacted C IVS RNA is systematically overestimated. The magnitude of the error, however, appears to be small. When the L – 15 → C′ → L – 19 reaction was analyzed at pH 9.0, we found that at no time did the fraction of circular RNA, C′/[C′ + (L – 15) + (L – 19)], exceed 0.1 (Zaug et al., 1984). Even if this proportion of C′ IVS were present at all time points in the experiment of Figure 5, it would result in a 10% overestimation of the amount of C IVS RNA (i.e., C + C′ > 1.1C) only when the reaction had gone 50% or more toward completion.

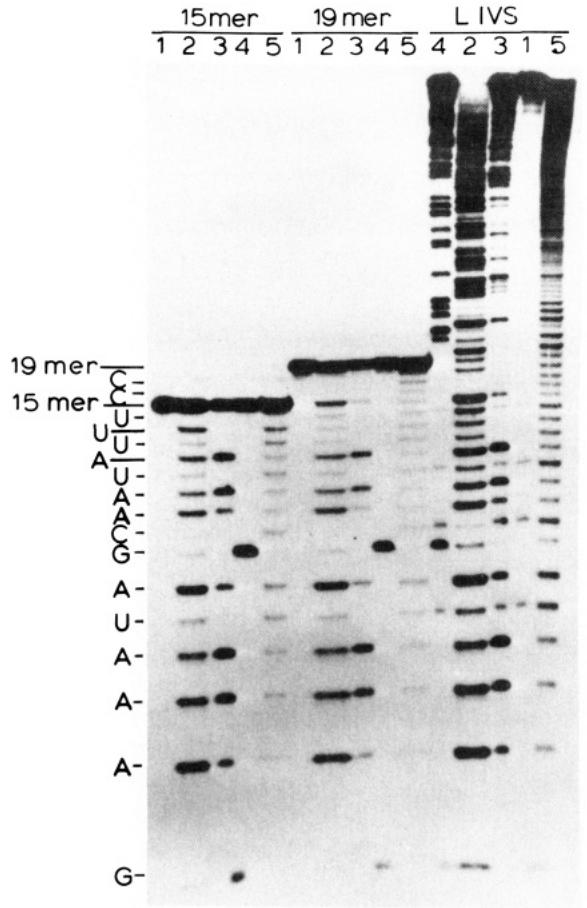


FIGURE 4: Sequence of 5′-end-labeled 19-mer compared to the 15-mer and the L IVS RNA. End-labeled L IVS RNA was incubated for 1 h under cyclization conditions (10 mM $MgCl_2$, 50 mM CHES, pH 9.0; 42 °C). The resulting 15-mer and 19-mer were purified by electrophoresis on a 20% polyacrylamide–7 M urea sequencing gel followed by the crush-and-soak procedure. (Lanes 1) No incubation. (Lanes 2) RNase Phy M, cleaves after A and U. (Lanes 3) RNase U_2, cleaves after A. (Lanes 4) RNase T_1, cleaves after G. (Lanes 5) Limited alkaline hydrolysis. Products were separated by electrophoresis in a 20% polyacrylamide–7 M urea gel.

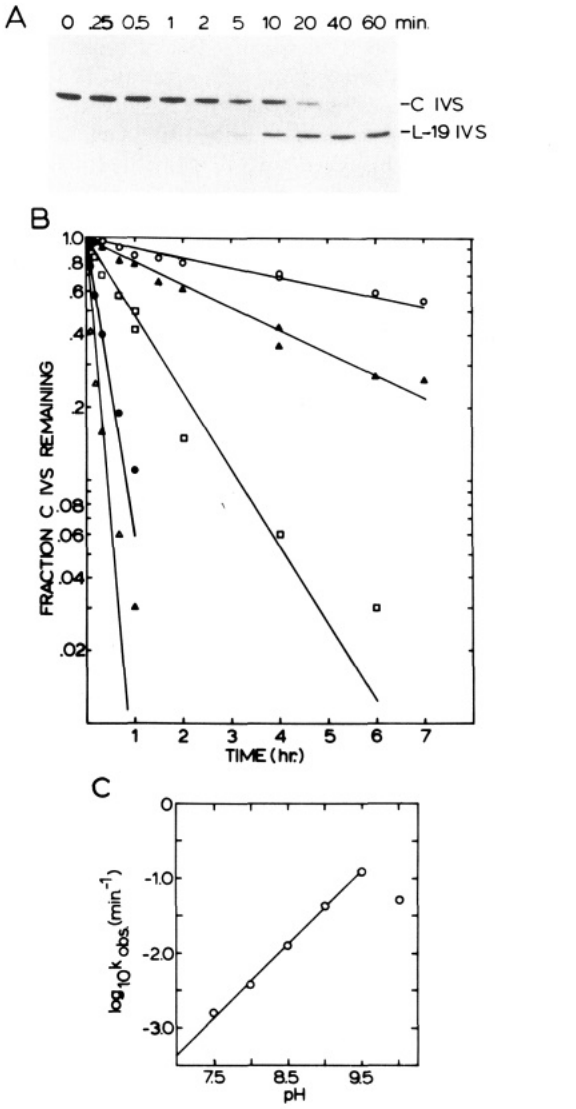


FIGURE 5: Effect of pH on C IVS RNA reopening by site-specific hydrolysis. (A) Sample of primary data. C IVS RNA, uniformly labeled with ^{32}P, was incubated at 42 °C in 10 mM $MgCl_2$, 200 mM NaCl, and 50 mM HEPPS, pH 9.0, for the indicated times. An autoradiogram of the 4% polyacrylamide–8 M urea gel is shown. (B) Semilog plot showing circle reopening as a function of time. (○) pH 7.5; (▲) pH 8.0; (□) pH 8.5; (●) pH 9.0; (△) pH 9.5. HEPPS buffer (pK_a = 8.0) was used for pH 7.5–9.0 and CHES (pK_a = 9.3) for pH 9.5. (C) Observed first-order rate constants for C IVS RNA reopening, obtained from the slopes of the lines in (B), are plotted as a function of pH.

(1980) have found that *E. coli* 5S rRNA undergoes a major conformational change when the pH is increased from 7 to 8.

Mechanism of Site-Specific Hydrolysis. The circular IVS RNA undergoes hydrolysis at the phosphodiester bond that was formed during cyclization (Zaug et al., 1984). The resulting linear RNA (L – 15 IVS) has 5′-phosphate and 3′-hydroxyl termini, in which respect the reaction resembles a reversal of cyclization. The reaction requires the folded structure of the RNA; it does not occur at high temperature or in the presence of 8 M urea (Zaug et al., 1984). We have now examined the kinetics of the reopening reaction and find that it is first order with respect to hydroxide ion concentration in the pH range 7.5–9.5. The most straightforward interpretation of these results is that the reaction involves nucleophilic attack of OH^- directly on the phosphate that joins G_{414} and A_{16}. An alternate explanation is that some residue or residues on the RNA are deprotonated as the pH is increased and that the deprotonation is required for the reaction to occur. The data of Figure 5C do not allow us to rule out such a possibility, as long as the pK_a of the hypothetical titratable group is >9.5 [cf. Lazarus et al. (1980)]. However, we note that the rate of the cyclization reaction is independent of pH over the same range that the rate of hydrolysis reaction increases linearly with pH. We think it unlikely that the hydrolysis reaction would require participation of some titratable group on the RNA that was not required for the cyclization reaction. We therefore favor and plan to test the hypothesis that circle reopening occurs by direct attack of OH^- at the ligation junction.

The rate of hydrolysis of the G_{414}–A_{16} phosphodiester bond in the C IVS is extremely high compared to that of other phosphate diesters (Table I). It is 10^{12}-fold greater than that estimated for dimethyl phosphate at the same temperature and is 10^3–10^4-fold greater than that of the strained five-membered cyclic phosphate esters, ethylene phosphate and uridine cyclic 2′,3′-phosphate. It is at least 10^3-fold and perhaps as much as 10^5-fold greater than that of an average phosphodiester bond in RNA. This difference becomes much more impresive when one notes that normal alkaline hydrolysis of RNA is greatly facilitated by the proximity of the 2′-OH which, when deprotonated, readily attacks the nearby phosphate, resulting in a cyclic 2′,3′-phosphate. Hydrolysis yielding a 5′-phosphate,

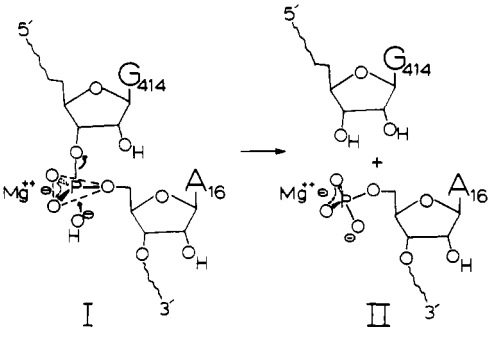
Table I: Rate Constants for the Alkaline Hydrolysis of Selected Phosphate Diesters[a]

compound	T (°C)	k (min^{-1} M^{-1})
dimethyl phosphate	25	2×10^{-10} [b,c]
	42	2×10^{-9} [b,c]
ethylene phosphate	25	3×10^{-2} [b]
RNA	27	2×10^{-2} [d]
uridine cyclic 2',3'-phosphate	30	3×10^{-1} [e]
	40	5×10^{-1} [e]
poly(A) + 0.2 mM Mg^{2+}	64	<1 [f]
G_{414}–A_{16} in C IVS RNA	42	2×10^{3} [g]

[a] Accurate data at a common temperature and ionic strength are not available. Available rate constants determined at temperatures close to 42 °C are given. [b] Kumamoto et al. (1956). [c] Corrected for the fraction of hydrolysis via P–O bond cleavage (0.1) according to Haake & Westheimer (1961). [d] Bock (1967). [e] Abrash et al (1967). [f] Butzow & Eichhorn (1965). [g] This work.

the specificity seen with the C IVS RNA reaction, is not normally observed. Mg^{2+} catalysis of phosphate ester hydrolysis has been noted previously, but not of the magnitude reported here. In a study of intramolecular nucleophilic catalysis of phosphate diester hydrolysis, Steffens et al. (1973) reported 24-fold rate enhancement of P–O bond cleavage at saturating Mg^{2+} concentration. As another example, the rate of hydrolysis of Poly(A) in the presence of Mg^{2+} is modest, even at 64 °C (Table I).

What might account for the extraordinary rate of hydrolysis of the cyclization junction of the C IVS? We prefer the idea that the structure of the RNA, a structure dependent upon Mg^{2+}, forces the phosphate to be distorted away from the normal tetrahedral geometry toward a trigonal bipyrimidal transition state, with one apical position unoccupied and therefore susceptible to nucleophilic attack (I). The degree



of bond strain would be considerably greater than that of the five-membered ring systems listed in Table I. In addition, the specific coordination of Mg^{2+} to the phosphate would enhance the electrophilicity of the phosphorus atom. Coordination of Mg^{2+} to one or more nucleotides, perhaps involving a substituent of a base as one of the ligands as in the Pb^{2+} binding site of tRNA (Brown et al., 1983; Rubin & Sundaralingam, 1983), could provide the required specificity. Finally, the metal ion could stabilize the presumed pentacovalent intermediate and/or transition states as described by Steffens et al. (1973, 1975) and Ikenaga & Inoue (1974). Reaction would occur by in-line attack of OH^-, an S_N2-type mechanism resulting in inversion of configuration (II). The chemistry would resemble that of the hydrolysis of RNA by pancreatic ribonuclease (Usher et al., 1970), with the important difference that the nucleophile is OH^- in C IVS hydrolysis and the ribose 2'-OH in RNase-catalyzed hydrolysis. These ideas are speculative but chemically reasonable.

If the strained phosphate idea is correct, IVS RNA cyclization should be energetically unfavorable, because it involves formation of the strained phosphate. Recent measurements indicate that cyclization is unfavorable, being accompanied by an increase in free energy of ~6 kcal/mol (Sullivan & Cech, 1985).

RNA-catalyzed hydrolysis of RNA is also found in the RNase P system (Guerrier-Takada et al., 1983). As in the self-catalyzed hydrolysis reaction described here, RNase P cleavage of tRNA precursors is site specific and produces 5'-phosphate and 3'-hydroxyl termini. The pH dependence of the *Bacillus subtilis* RNase P reaction has been determined (Marsh & Pace, 1985). While the reaction rate increases linearly with pH in the range pH 6–8, the amount of the increase is less than expected for a model of direct attack by OH^-. The *E. coli* RNase P reaction shows no pH dependence between about pH 5 and pH 9 for either the RNA subunit alone or the holoenzyme, RNA plus protein (S. Altman, personal communication). The implication of these results is that the nucleophile may be H_2O rather than OH^-.

Reaction Sites. We have describe two new sites of cleavage of the L IVS RNA in addition to the cleavage at position 15 that accompanies the major cyclization reaction. One cleavage site was around position 26. The structure of the resulting oligonucleotide has not been determined. If it has a 3'-hydroxyl terminus, it contains 26 nucleotides and results from cleavage at G_{26}–G_{27}. If it has a 3'-phosphate terminus, it is probably a 27-nucleotide fragment, in which case it results from cleavage at G_{27}–A_{28}. We have not seen circles or reopened circles corresponding to a size of 388 nucleotides and therefore have no reason to believe that cyclization accompanies this cleavage reaction. Production of this fragment was enhanced in Tris buffer, reaching a maximum at pH 8.5–9.0, the pH at which the amino group of Tris is fully neutralized. On the basis of these observations, it seems possible that Tris is acting as a nucleophile and that the specificity of the reaction for the nucleotide 26–27 region of the RNA reflects some unusual structural features of that region. That region has attracted our attention before; it appears to undergo a structural change when the L IVS undergoes cyclization (Inoue & Cech, 1985). Thus, while it is unlikely that this cleavage reaction has any biological relevance, the Tris-induced cleavage may prove to be a useful probe for structure in this region of the molecule.

The other new L IVS cleavage reaction, at U_{19}–U_{20}, is probably concomitant with cyclization at U_{20}. This has been difficult to prove, since the relative amount of the reaction is minor and a circular RNA (C' IVS) resulting from cyclization at U_{20} would be only 4 nucleotides smaller than the C IVS. Closely spaced doublets of circular IVS RNAs have been resolved on long polyacrylamide gels by using tritium fluorography to obtain high resolution and may correspond to the C and C' species [Figure 3 of Price et al. (1985)]. Moreover, the occurrence of L – 19 IVS RNA with U_{20} at its 5' end and G_{414} at its 3' end has been proven (Zaug et al., 1984); such molecules appear to result from reopening of C' IVS RNA.

We have noted a similarity between IVS cyclization and the first step in pre-rRNA splicing: both reactions involve attack by a G-OH, cleavage of UpA and formation of a GpA linkage (Zaug et al., 1983). Finding cyclization that involves cleavage of UpU means that UpA is not mandatory for such reactions. The preference of UpA over UpU for cyclization could reflect the relative probability of two alternate structures of the RNA, with UpA positioned in the active site in one structure and UpU in the site in the other structure. Alternatively, there could be a single structure in which the UpA and the UpU have different reactivity due to their position in the molecule or their sequence. We are now in the process

of using in vitro mutagenesis to further explore the requirements for sequence and structure at the splicing and cyclization sites.

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Registry No. OH^-, 14280-30-9; GpA, 6554-00-3; GpU, 4785-07-3.

REFERENCES

Abrash, H. I., Cheung, C.-C. S., & Davis, J. C. (1967) *Biochemistry 6*, 1298–1303.

Baes, C. F., Jr., & Mesmer, R. E. (1976) *The Hydrolysis of Cations*, pp 95–98, Wiley, New York.

Bock, R. M. (1967) *Methods Enzymol. 12A*, 218–221.

Brehm, S. L., & Cech, T. R. (1983) *Biochemistry 22*, 2390–2397.

Brown, R. S., Hingerty, B. E., Dewan, J. C., & Klug, A. (1983) *Nature (London) 303*, 543–546.

Bruce, A. G., & Uhlenbeck, O. C. (1978) *Nucleic Acids Res. 5*, 3665–3677.

Butzow, J. J., & Eichhorn, G. L. (1965) *Biopolymers 3*, 95–107.

Cech, T. R. (1983) *Cell (Cambridge, Mass.) 34*, 713–716.

Cech, T. R., Zaug, A. J., & Grabowski, P. J. (1981) *Cell (Cambridge, Mass.) 27*, 487–496.

Donis-Keller, H. (1980) *Nucleic Acids Res. 8*, 3133–3142.

Donis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527–2538.

Garriga, G., & Lambowitz, A. M. (1983) *J. Biol. Chem. 258*, 14745–14748.

Garriga, G., & Lambowitz, A. M. (1984) *Cell (Cambridge, Mass.) 39*, 631–641.

Grabowski, P. J., Zaug, A. J., & Cech, T. R. (1981) *Cell (Cambridge, Mass.) 23*, 467–476.

Greer, C. L., Peebles, C. L., Gegenheimer, P., & Abelson, J. (1983) *Cell (Cambridge, Mass.) 32*, 537–546.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., & Altman, S. (1983) *Cell (Cambridge, Mass.) 35*, 849–857.

Haake, P. C., & Westheimer, F. H. (1961) *J. Am. Chem. Soc. 83*, 1102–1109.

Haydock, K., & Allen, L. C. (1985) *Prog. Clin. Biol. Res. 172A*, 87–98.

Ikenaga, H., & Inoue, Y. (1974) *Biochemistry 13*, 577–582.

Inoue, T., & Cech, T. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 648–652.

Kao, T. H., & Crothers, D. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3360–3364.

Konarska, M., Filipowicz, W., Domdey, H., & Gross, H. L. (1981) *Nature (London) 293*, 112–116.

Konarska, M., Filipowicz, W., & Gross, H. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1474–1478.

Kruger, K., Grabowski, P. J., Zaug, A. J., Sands, J., Gottschling, D. E., & Cech, T. R. (1982) *Cell (Cambridge, Mass.) 31*, 147–157.

Kumamoto, J., Cox, J. R., Jr., & Westheimer, F. H. (1956) *J. Am. Chem. Soc. 78*, 4858–4860.

Lazarus, R. A., Benkovic, P. A., & Benkovic, S. J. (1980) *J. Chem. Soc., Perkin Trans. 2*, 373–379.

Marsh, T. L., & Pace, N. R. (1985) *Science (Washington, D.C.) 229*, 79–81.

Padgett, R. A., Konarska, M. M., Grabowski, P. J., Hardy, S. F., & Sharp, P. A. (1984) *Science (Washington, D.C.) 225*, 898–903.

Peattie, D. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1760–1764.

Price, J. V., Kieft, G. L., Kent, J. R., Sievers, E. L., & Cech, T. R. (1985) *Nucleic Acids Res. 13*, 1871–1889.

Rubin, J. R., & Sundaralingam, M. (1983) *J. Biomol. Struct. Dyn. 1*, 639–646.

Ruskin, B., Krainer, A. R., Maniatis, T., & Green, M. R. (1984) *Cell (Cambridge, Mass.) 38*, 317–331.

Silber, R., Malathi, V. G., & Hurwitz, J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3009–3013.

Steffens, J. J., Sampson, E. J., Siewers, I. J., & Benkovic, S. J. (1973) *J. Am. Chem. Soc. 95*, 936–938.

Steffens, J. J., Siewers, I. J., & Benkovic, S. J. (1975) *Biochemistry 14*, 2431–2440.

Sullivan, F. X., & Cech, T. R. (1985) *Cell (Cambridge, Mass.) 42*, 639–648.

Tabak, H. F., Van der Horst, G., Osinga, K. A., & Arnberg, A. C. (1984) *Cell (Cambridge, Mass.) 39*, 623–629.

Ts'o, P. O. P. (1974) in *Basic Principles in Nucleic Acid Chemistry*, Vol. I, pp 462–463, Academic Press, New York.

Usher, D. A., Richardson, D. I., Jr., & Eckstein, F. (1970) *Nature (London) 228*, 663–665.

Van der Horst, G., & Tabak, H. F. (1985) *Cell (Cambridge, Mass.) 40*, 759–766.

Wallace, J. C., & Edmonds, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 950–954.

Zaug, A. J., Grabowski, P. J., & Cech, T. R. (1983) *Nature (London) 301*, 578–583.

Zaug, A. J., Kent, J. R., & Cech, T. R. (1984) *Science (Washington, D.C.) 224*, 574–578.